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<p>(54) Title: INTERLEUKIN CONVERTING ENZYME (ICE) AND CENTRAL NERVOUS SYSTEM DAMAGE (57) Abstract The invention relates to methods of treating central nervous system damage. This includes methods of treating ALS using a mutant ICE gene and methods of treating head trauma injuries by ICE inhibition. The invention also relates to transgenic non-human animals comprising a mutant ICE gene and a mutant SOD gene. These transgenic animals exhibit attenuated symptoms of ALS. This invention also relates to methods of using the transgenic animals to screen for compounds to treat ALS.</p>		

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Interleukin Converting Enzyme (ICE) and Central Nervous System Damage

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Part of the work performed during the development of this invention was supported by U.S. Government funds. The U.S. Government may have certain rights in this invention.

Background of the Invention

15 *Field of the Invention*

The invention is generally in the field of molecular biology as related to the control of programmed cell death and treatment of disease. The invention further relates to treatment of amyotrophic lateral sclerosis and head trauma injury.

20 *Related Art*

Amyotrophic Lateral Sclerosis (ALS)

25 Amyotrophic lateral sclerosis (ALS), is a progressive age-dependent disease involving degeneration of motor neurons in the brain, brain stem and spinal cord. Both familial and sporadic forms of this disease exist. In the most frequent form of ALS, patients evidence both lower motor neuron impairment (including muscular atrophy and weakness) as well as upper motor neuron

damage. The principal pathologic changes are loss of motor neurons and their axons, with very little gliotic reaction. Related variants of ALS include progressive bulbar palsy, progressive muscular atrophy and primary lateral sclerosis. (Robbins *et al.*, *Basic Pathology*, W.B. Saunder Co. (1987))

5 ALS is characterized by neuronal cell death. Little is known about the triggering mechanism responsible for executing this cell death in ALS. Although ALS has been included in a list of diseases associated with increased apoptosis (Thompson, C.B., *Science* 267:1456-1462 (1995), there has been no direct evidence in the art to indicate that such is actually the case.

10 Mutations in the Cu/Zn superoxide dismutase (SOD-1) gene have been shown to be responsible for some of the familial forms of ALS (Rosen, D.R., *et al.*, *Nature* 362:59-62 (1993)). Additionally, evidence described by Rothstein *et al.* (*Proc. Natl. Acad. Sci. USA* 91:4155-9 (1994)) demonstrated that down regulation of SOD-1 activity using antisense SOD-1 *in vitro* promotes apoptosis
15 in a neuronal cell line. Cell death in the neuronal cell line was mediated in part by the activation of the Interleukin-1 β converting enzyme (ICE), and by binding of endogenously produced mature IL-1 β to its receptor (Troy, C.M., *et al.*, *Proc. Natl. Acad. Sci. USA* 93:5635-40 (1996)). Therefore, a better understanding of the role of cell death and what triggers such death in ALS would lead to a more
20 rational treatment and possible cure for the disease.

Traumatic Brain Injury (TBI)

 Traumatic injury is the third leading cause of death in the western world, superseded only by cancer and heart disease. Half of traumatic deaths are directly attributed to brain injury (Waxweiler, R.J., *et al.*, *J. Neurotrauma* 12:509-516
25 (1995); Department of Health and Human Services, *Interagency Head Injury Task Force Report* (1989)). Despite the societal impact of traumatic brain injury (TBI), little is known regarding the basic mechanistic pathways by which it mediates cell death. Traditional notion attributes cell death to necrosis as the major mechanism following TBI (Shapira, Y., *et al.*, *Crit. Care Med.* 16:258-265

(1988); Dietrich, W.D., *et al.*, *Acta Neuropathol.* 87:250-258 (1994)). This view has been recently challenged with the detection of apoptotic cells in experimental brain injury models (Rink, A., *et al.*, *Am. J. Pathol.* 147:1575-1583 (1995); Colicos, M.A. & Dash, P.K., *Brain Res.* 739:120-131 (1996); Yakovlev, A.G. *et al.*, *J. Neurosci.* 17:7415-7424 (1997); Sinson, G., *et al.*, *J. Neurosurg.* 86:511-518 (1997)), as well as in humans following head trauma (Thomas, L.B., *et al.*, *Exp. Neurol.* 133:265-272 (1995)). Understanding the pathways mediating posttraumatic apoptosis might lead to novel approaches to rational pharmacotherapy of TBI.

10 *Programmed Cell Death*

Apoptosis, also referred to as programmed cell death or regulated cell death, is a process by which organisms eliminate unwanted cells. Such cell death occurs as a normal aspect of animal development as well as in tissue homeostasis during aging and in disease (Glucksmann, A., *Biol. Rev. Cambridge Philos. Soc.* 26:59-86 (1950); Ellis *et al.*, *Dev.* 112:591-603 (1991); Vaux *et al.*, *Cell* 76:777-779 (1994); Thompson, C.B., *Science* 267:1456-1462 (1995))). Programmed cell death can also act to regulate cell number, to facilitate morphogenesis, to remove harmful or otherwise abnormal cells and to eliminate cells that have already performed their function. Additionally, programmed cell death is believed to occur in response to various physiological stresses such as hypoxia or ischemia. The morphological characteristics of apoptosis include plasma membrane blebbing, condensation of nucleoplasm and cytoplasm and degradation of chromosomal DNA at inter-nucleosomal intervals. (Wyllie, A. H., in *Cell Death in Biology and Pathology*, Bowen and Lockshin, eds., Chapman and Hall (1981), pp. 9-34).

Apoptosis is achieved through an endogenous mechanism of cellular suicide (Wyllie, A. H., in *Cell Death in Biology and Pathology*, Bowen and Lockshin, eds., Chapman and Hall (1981), pp. 9-34) and occurs when a cell activates its internally encoded suicide program as a result of either internal or

external signals. The suicide program is executed through the activation of a carefully regulated genetic program (Wyllie, A.H., *et al.*, *Int. Rev. Cyt.* 68: 251 (1980); Ellis, R.E., *et al.*, *Ann. Rev. Cell Bio.* 7: 663 (1991); Yuan, Y. *Curr. Op. Cell. Biol.* 7:211-214 (1995)). In many cases, gene expression appears to be required for apoptosis, since cell death can be prevented by inhibitors of RNA or protein synthesis (Cohen *et al.*, *J. Immunol.* 32:38-42 (1984); Stanicic *et al.*, *Invest. Urol.* 16:19-22 (1978); Martin *et al.*, *J. Cell Biol.* 106:829-844 (1988).

Acute and chronic dysregulation of cell death is believed to lead to a number of major human diseases (Barr *et al. Biotech.* 12:487-493 (1995); Thompson C.B., *Science* 267:14561462 (1995)). These diseases include but are not limited to malignant and pre-malignant conditions, neurological and neurodegenerative disorders, heart disease, immune system disorders, intestinal disorders, kidney disease, aging, viral infections and AIDS.

Malignant and pre-malignant conditions may include solid tumors, B cell lymphomas, chronic lymphocytic leukemia, prostate hypertrophy, preneoplastic liver foci and resistance to chemotherapy. Neurological disorders may include stroke, Alzheimer's disease, amyotrophic lateral sclerosis, prion-associated disorder and ataxia telangiectasia. Heart disease may include ischemic cardiac damage and chemotherapy-induced myocardial suppression. Immune system disorder may include AIDS, type I diabetes, lupus erythematosus, Sjogren's syndrome and glomerulonephritis. Intestinal disorder may include dysentery, inflammatory bowel disease and radiation- and HIV-induced diarrhea. Kidney disease may include polycystic kidney disease and anemia/erythropoiesis. Specific references to many of these pathophysiological conditions as involving dysregulated apoptosis can be found in Barr *et al. Id.* - Table I.

Interleukin-1- β Converting Enzyme (ICE or Caspase) Family

Mechanistically, apoptotic cell death is mediated by a family of cysteine proteases known as caspases (Alnemri, E.S., *et al.*, *Cell* 87:171 (1996)). Caspases are mammalian homologues of the *C. elegans* death gene product CED-

3 (Yuan, J. & Horvitz, H.R., *Dev. Biol.* 138:33-41 (1990); Yuan, J., Shaham, *et al.*, *Cell* 75:641-652 (1993)) which execute, together with CED-4, apoptotic cell death in the nematode. Interleukin-1 β converting enzyme (ICE; caspase-1), the first identified member of the mammalian caspase family, is a cysteine protease responsible for the activation of pro-IL- β (Yuan, J., Shaham, *et al.*, *Cell* 75:641-652 (1993); Ceretti, D.P., *et al.*, *Science* 256:97-100 (1992); Miura, M., *Cell* 75:653-660 (1993)). The involvement of ICE in apoptosis has been demonstrated in a variety of experimental paradigms (Miura, M., *Cell* 75:653-660 (1993); Gagliardini, V., *et al.*, *Science* 263:826-828 (1994); Los, M., *et al.*, *Nature* 375:81-83 (1995); Enari, M., *et al.*, *Nature* 380:723-726 (1996)). ICE activation, as demonstrated by the detection of mature IL-1 β b, has been identified during apoptosis both *in vitro* as well as *in vivo* (Hogquist, K.A., *et al.*, *Proc. Natl. Acad. Sci. USA* 88:8485-8489 (1991); Zychlinsky, A., *et al.*, *J. Clin. Invest.* 94:1328-1332 (1994); Miura, M., *et al.*, *Proc. Natl. Acad. Sci. USA* 92:8318-8322 (1995); Hara, H., *et al.*, *Proc. Natl. Acad. Sci. USA* 94:2007-2012 (1997); Hara, H., *et al.*, *J. Cereb. Blood Flow Metab.* 17:370-375 (1997)). It has previously been shown that endogenously produced mature IL-1 β b, processed following ICE activation, plays an important role in apoptosis (Friedlander, R.M., *et al.*, *J. Exp. Med.* 184:717-724 (1996); Troy, C.M., *et al.*, *Proc. Natl. Acad. Sci. USA* 93:5635-5640 (1996)).

ICE is a cytoplasmic cysteine protease responsible for proteolytic processing of pro-interleukin-1 β (31 kDa) into its active form (17 kDa) (Thornberry, N.A., *Nature* 356:768-774 (1992), Cerretti, D.P., *et al.*, *Science* 256:97-100 (1992)). ICE is synthesized as a precursor of 45kDa which is proteolytically cleaved during activation to generate two subunits of 22kDa (p20) and 10kDa (p10) (Thornberry, N.A., *et al.*, *Nature* 356:768-774 (1992)).

ICE is a member of a large family of apoptotic gene products. The ICE family (Caspase family) comprises an increasing number of cysteine proteases involved in cytokine maturation and apoptosis (Yuan, J., *Curr. Opin. in Cell Biology* 7:211-214 (1995)). To date, ten ICE homologs of human origin have

been published and the family members are now also referred to as "caspases." (Alnemri *et al.*, *Cell* 87:171 (1996)). ICE is referred to as caspase-1 (CASP-1). Frequently, the murine caspases may be found to have the designation mCASP. (Van de Craen *et al.*, *FEBS Lett.* 401:61-69 (1997))

5 The mammalian ICE/CED-3 family includes eight members ICE, TX/ICE_{re}II/ICH-2, ICE_{re}III, ICH-1/NEDD2, CPP32/Yama/Apopain, MCH2, MCH-3/ICE-LAP3/MCH-2 and ICH-3 (Kumar *et al.*, *Genes Dev.* 8:1613-1626 (1994); Fernandes-Alnemri, *et al.*, *J. Biol. Chem.* 269:30761-30764 (1994); Fernandez-Alnemri *et al.*, *Cancer Res.* 55:2737-2742 (1995); Fernandes-Alnemri
10 *et al.*, *Cancer Res.* 55:6045-6052 (1996); Wang *et al.*, *Cell* 78:739-750 (1994); Faucheu, *et al.*, *EMBO J.* 14:1914-1922 (1995); Tewari & Dixit, *J. Biol. Chem.* 270:3255-3260 (1995); Kamens *et al.*, *J. Biol. Chem.* 270:15250-15256 (1995); Munday, N.A., *et al.*, *J. Biol. Chem.* 270:15870-15876 (1995); Duan, H.J., *et al.*, *J. Biol. Chem.* 271:1621-1625 (1996); Lippke, J.A., *et al.*, *J. Biol. Chem.*
15 271:1825-1828 (1996)). All of the above have more recently been designated as caspases. A comparison of ICE and ced-1 is shown in Figure 1A-1B.

X-ray crystallography analysis of three dimensional structure of ICE showed that ICE is a dimer of activated ICE p20 and p10 subunits (Wilson, K.P., *et al.*, *Nature* 370:270-275 (1994); Walker, N.P.C., *et al.*, *Cell* 78:343-352 (1994)). Activated ICE can cleave the inactive ICE precursor; however, *in vitro* synthesized ICE precursor cannot cleave itself (Thornberry, N.A., *et al.*, *Nature* 356:768-774 (1992)), suggesting that ICE may need to be activated by another protease *in vivo*.

25 The amino acid sequence of ICE shares 29% identity with *C. elegans* cell death gene product Ced-3 (Yuan *et al.*, *Cell* 75:641-752 (1993)) which suggests that ICE may play a role in controlling mammalian apoptosis. In this regard, it has been demonstrated that ICE-mediated endogenously produced mature IL-1 β plays an important role in a variety of cell death paradigms (Friedlander, R.M., *et al.*, *J. Exp. Med.* 184:717-724 (1996)).

Expression of ICE in a number of mammalian cell lines induces apoptosis (Miura *et al.*, *Cell* 75:653-660 (1993); Wang *et al.*, *Cell* 87:739-750 (1994)). Microinjection of an expression vector of crmA, a cowpox virus gene encoding a serpin that is a specific inhibitor of ICE, prevents the death of neurons from the dorsal root ganglia and ciliary ganglia, when such death is induced by trophic factor deprivation (Gagliardini *et al.*, *Science* 263:826-828 (1994); Li *et al.*, *Cell* 80:401-411 (1995); Allsopp *et al.*, *Cell* 73:295-307, (1993)). Expression of crmA can also suppress apoptosis induced by TNF- α and Fas (Enari *et al.*, *Nature* 375:78-81 (1995); Los *et al.*, *Nature* 375:81-83 (1995); Kuide *et al.*, *Science* 267:2000-2002 (1995); Miura *et al.*, *Proc. Natl. Acad. Sci. USA* 92:8318-8322 (1995)). These experiments suggest that the members of the ICE family (Caspase family) play important roles in controlling mammalian apoptosis. These results did not indicate, however, which member of the ICE family (Caspase family) is critical for cell death since CrmA may cross-inhibit other members of the ICE family.

The control of apoptosis in mammals is much more complex than that in *C. elegans* where function of one *ced-3* gene controls all programmed cell death (Ellis & Horvitz, *Cell* 44:817-829 (1986)). In contrast to *C. elegans*, multiple proteases may be involved in regulation of programmed cell death (apoptosis) in mammals. This hypothesis is supported by many *in vitro* studies. For instance, peptide inhibitors of ICE such as YVAD-cmk inhibit Fas induced apoptosis but require much higher doses than that for inhibiting ICE (Enari *et al.*, *Nature* 375:78-81 (1995)), suggesting that inhibition of additional ICE-like protease(s) is required for complete inhibition of Fas induced apoptosis. Similarly, Ac-DEVD-CHO, a peptide inhibitor of CPP32/Yama/Apopain, inhibits poly(ADP-ribose) polymerase (PARP) cleavage at a dose of 1 nM but requires 1 μ M to cause 50% inhibition of apoptosis in an cell-free system (Nicholson, D.W., *et al.*, *Nature* 376:37-43 (1995)), suggesting that inhibition of protease(s) other than CPP32/Yama/Apopain is required for complete inhibition of apoptosis in this system. Furthermore, inhibitors that are known not to have effects or have

little effects on ICE-like cysteine proteases such as cysteine protease inhibitors trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E64) and leupeptin, calpain inhibitors I and II, and serine protease inhibitors diisopropyl fluorophosphate and phenylmethylsulfonyl fluoride, were found to inhibit apoptosis induced by T cell receptor binding-triggered apoptosis (Sarin *et al.*, *J. Exp. Med.* 178:1693-1700 (1993)). This suggests that not only cysteine proteases but also serine proteases may play important roles in mammalian cell apoptosis.

Additionally, ICE may also be involved in γ -irradiation induced cell death in concanavalin A (conA)-stimulated splenocytes (Tamura *et al.*, *Nature* 376:596-599 (1995)). Expression of ICE is induced in splenocytes stimulated by conA and induction of *Ice* expression enhances the susceptibility of mitogen activated T cells to cell death induced by γ -irradiation and DNA-damaging chemotherapeutic agents such as adriamycin or etoposide induced cell death.

Knowing the genes and substrates involved in the ICE pathway and effects of altering or eliminating expression of apoptotic proteins such as ICE or ICH-3 leads to means for modulating (i.e. increasing or decreasing) cell death thereby altering apoptosis. A better understanding of the apoptosis pathways and specific gene products such as ICE can also lead to development of assays for agents which may affect the apoptotic process, and thereby lead to therapies for disease treatments. Interventions may include, *inter alia*, agents which affect the activities of the gene products (e.g. agents which block receptors, inhibit or stimulate enzymatic activity), modulation of the gene product using gene-directed approaches such as anti-sense oligodeoxynucleotide strategies, transcriptional regulation and gene therapy (Karp *et al.*, *Cancer Res.* 54:653-665 (1994)). Therefore, apoptosis should be amenable to therapeutic intervention. In this regard, one may either stimulate or inhibit the process depending upon whether one wants to increase or decrease the rate of programmed cell death.

Transgenic Animals

Techniques that allow foreign DNA sequences to be introduced into the

mammalian germ line have been developed in mice. *See Manipulating the Mouse Embryo* (Hogan *et al.*, eds., 2d ed., Cold Spring Harbor Press, 1994) (ISBN 0-87969-384-3). At present, one route of introducing foreign DNA into a germ line entails the direct microinjection of a few hundred linear DNA molecules into a pronucleus of a fertilized one-cell egg. Microinjected eggs may then be subsequently transferred into the oviducts of pseudo-pregnant foster mothers and allowed to develop. It has been reported by Brinster *et al.* (1985), that about 25% of the mice that develop inherit one or more copies of the micro-injected DNA.

In addition to transgenic mice, other transgenic animals have been made. For example, transgenic domestic livestock, such as pigs, sheep, and cattle. Once integrated into the germ line, the foreign DNA may be expressed in the tissue of choice at high levels to produce a functional protein. The resulting animal exhibits the desired phenotypic property resulting from the production of the functional protein.

With so many members in the ICE/CED-3 family, it is important to determine the ICE/CED-3 family member's functions individually. Transgenic mice are an ideal model for accomplishing this by generating mutations in the genes of interest, or knocking out a particular gene. Using such models, it has already been shown that mice deficient in ICE develop normally but are resistant to endotoxic shock induced by lipopolysaccharide (LPS). This can be attributed to their defect in production of mature IL-1 β (Li *et al.*, *Cell* 80:401-411 (1995); Kuida *et al.*, *Science* 267:2000-2003 (1995)). Furthermore, ICE deficient thymocytes from knockout mice undergo apoptosis normally when stimulated with dexamethasone and γ -irradiation but are partially resistant to Fas induced apoptosis (Kuida *et al.*, *Science* 267:2000-2003 (1995)), suggesting that ICE plays an important role in Fas but not dexamethasone and γ -irradiation induced apoptosis in thymocytes.

Generation of mutant mice by gene targeting technique, making crosses between mice of potential candidate genes, should provide vital information about the genetic and biochemical pathways of apoptosis. In this regard, over the

last several years, transgenic animals containing specific genetic defects, e.g., those resulting in the development of, or predisposition to, various disease states, have been made. These transgenic animals can be useful in characterizing the effect of such a defect on the organism as a whole, and developing pharmacological treatments for these defects. Obtaining transgenic animals in which specific genes and proteins of the apoptotic pathway are altered or eliminated (e.g. knock-out mice) results in a better understanding of the regulation of programmed cell death.

A transgenic mouse has been made whose phenotype is similar to ALS (Gurney *et al.*, *Science* 264:17721775 (1994)). The trans-gene has a mutation in superoxide dismutase (SOD). These animals have age-dependent progressive motor weakness similar to ALS in humans. A different transgenic mouse has now been made that expresses a mutant of ICE, which is a dominant negative inhibitor of the ICE pathway. (Friedlander *et al.*, *J. Exp. Med.* 185:933-940 (1997)).

In light of the various biological roles of apoptosis in disease, there exists a need in the art to develop treatments addressed to modulating cell death in various pathological states, such as ALS. There also exists a need to develop transgenic animals, e.g., transgenic mice, wherein one can test the role of apoptosis in ALS. Finally, there also exists a need in the art to develop methods to test compounds directed to modifying the apoptotic condition using such transgenic animals.

Summary of the Invention

It has now been found that a dominant negative mutant of the cell death gene ICE (Caspase-1) significantly slows the symptomatic progression of ALS and delays mortality in a transgenic mouse model of ALS. This suggests the involvement of the ICE-like proteases in ALS progression and the therapeutic value of ICE inhibitors in the treatment of ALS in humans.

It has also been found that when a transgenic mouse expressing an ALS phenotype (SOD mutation) is crossed with a transgenic mouse having a mutant *ICE* gene, the resulting offspring have an attenuated form of ALS. This provides a new transgenic model for studying ALS and for testing new treatments for the disease.

Therefore, this invention satisfies a need in the art for finding a treatment for ALS and providing new animal models to study this disease.

The invention is first directed to a method for treating ALS. Preferably, the invention is directed to treating cell death during ALS and more preferably to treating neuronal cell death during ALS.

In an embodiment of this invention the treatment of ALS involves gene therapy to ameliorate the effects of the *ICE* gene. Preferably the gene therapy involves use of a mutant *ICE* gene comprising a DNA sequence encoding an amino acid sequence wherein the cysteine residue in the active site of ICE is replaced with a glycine. In a specific embodiment the replacement at the cysteine residue in the active site of the murine ICE is at amino acid 284 (C284G). More preferably, the gene sequence is found in plasmid pJ655 having ATCC accession number 209077 deposited in the American Type Culture Collection (10801 University Boulevard, Manassas, Virginia 20110-2209, USA, on May 28, 1997 under the Budapest Treaty) or is a degenerate variant of said mutant gene.

In another embodiment of the invention, treatment of ALS involves the use of protease inhibitors selected from the group consisting of N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD.FMK), acetyl-Tyr-Val-Ala-Asp-chloromethylketone (Ac-YVAD.CMK) (SEQ ID No. 26), N-benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone (z-DEVD.FMK) (SEQ ID No. 27) and Ac-YVAD-CHO (SEQ ID No. 28).

Another embodiment of the invention involves the treatment of traumatic brain injury by inhibition of the ICE cell death family. This may be done by use of ICE inhibitors or directly affecting the relevant gene as in the knockout mice

of the claimed invention. A further related embodiment of the invention is drawn to reducing the formation of reactive oxygen species (ROS) by inhibiting the ICE cell death family.

5 The invention is further directed to a non-human transgenic animal expressing the ALS phenotype that also contains a mutant *ICE* gene. Preferably the non-human animal is a mouse. Such mice exhibit an increased period of time in which the disease exists and therefore live longer than an ALS mouse not expressing the dominant negative mutant *ICE* gene.

10 In a preferable embodiment of this invention, the mutant *ICE* gene in the transgenic animal comprises DNA encoding an amino acid sequence wherein the cysteine residue in the active site of ICE is replaced with a glycine, e.g. in the mouse (C284G). More specifically, the mutant gene is found in plasmid pJ655 having ATCC accession number 209077 or is a degenerate variant of said mutant gene.

15 In additional embodiments, this invention provides a method of testing compounds affecting ALS by providing a non-human animal with ALS that also has a mutant *ICE* gene, wherein the animal exhibits an increased resistance to ALS. One administers a compound to be tested to the transgenic animal, and determines the effect of the compound on the mortality of the animal relative to
20 an animal with the SOD mutation but without the mutant *ICE* gene.

Brief Description of the Figures

Figures 1A-1B. The amino acid sequence of ced-3 and ICE genes.
(SEQ ID NOS: 1-2, 4 and 7-11) Figure 1A-1B contains a comparison of the
25 amino acid sequences of ced-3 from *C. elegans*, *C. briggsae* and *C. vulgaris* with hICE, mICE and mouse nedd-2. Amino acids are numbered at the right of each row. The lines indicate gaps resulting from obtaining optimal alignment of the sequences. Residues that are conserved among more than half of the proteins are boxed. Missense *ced-3* mutations are indicated above the comparison blocks

showing the residue in the mutant ced-3 and the allele name. Asterisks indicate potential aspartate self-cleavage sites in ced-3. Circles indicate known aspartate self-cleavage sites in hICE. Figure 1A-1B also includes the sequences of mutant ICE proteins wherein the C is replaced with a G (SEQ. ID. Nos. 1-2). As indicated on the figure, the mutation in the mouse gene encodes a protein having glycine rather than cysteine at position 284 (C284G) (SEQ ID NO:1). A similar mutation may be created in the human ICE, except the change is made at position 285 (C285G). (SEQ ID NO: 2)

Figure 2A-2D - DNA and amino acid sequence of wild-type and mutant murine ICE. Figure 2A is the amino acid sequence of wild-type murine ICE (SEQ ID NO: 4). Figure 2B is the DNA sequence of wild-type murine ICE (SEQ ID NO:5). Figure 2C is the amino acid sequence of the mutant murine ICE (SEQ ID NO:6). Figure 2D is the DNA sequence of the mutant murine ICE (SEQ. ID. No. 3).

Figure 3A-3C: Protection from permanent middle cerebral artery (MCA) occlusion-mediated infarct. Infarct protection in NSE-M17Z (black) was compared with wild-type (white) mice. Figure 3A. Neurological grading 30 minutes and 24 hours following occlusion. Neurological grading: 0=no neurological deficits; 1=failure to extend the right forepaw; 2=circling to the contralateral side; 3=loss of walking or righting reflex. Figure 3B. Infarct area assessed at 24 hours. Figure 2C. Regional cerebral blood flow (rCBF), and mean blood pressure (MBP) of wild type and transgenic mice during 30 minutes of ischemia ($p<0.01$).

Figure 4: Whole brain lysates of NSE-M17Z mice are deficient in processing pro-IL-1 β following systemic LPS (lipopolysaccharide) administration. LPS was injected intraperitoneally (10 μ g/gr body weight) and 2 hours before sacrifice (wild type n=4, NSE-M17Z n=5). PBS was injected as

a control (wild type n=3, NSE-M17Z n=6). Brains were dissected, and mature IL-1 β concentration was determined using an ELISA kit specific for mature IL-1 β . Results are expressed as means \pm SEM.

Figure 5: DNA damage in the lesioned hemisphere of wild-type mice.

5 Lane 1 shows the DNA size marker with 200 bp steps (M), lane 2 and 3 (T1 and T2) the DNA ladder prepared from right coronal sections 6 mm from frontal pole 24 hours after weight drop trauma, and lane 4 and 5 (S1 and S2) the DNA from the corresponding section of the right hemisphere of sham-operated animals. T1, T2, S1 and S2 were taken from different animals.

Figure 6: Trauma-induced elevation of mature IL-1 β levels in brain.

10 Empty columns: sham-operated animals, black columns: traumatized animals. Data are presented as mean \pm SEM (n = 5 or 6 in duplicate); *P<0.02 vs. contralateral hemisphere in traumatized animals; *P<0.008 vs. ipsilateral hemisphere in sham-operated animals. Mature IL-1 β quantification was performed as previously described using an ELISA kit (Genzyme, Cambridge, MA) (Hara, H., *et al.*, *Proc. Natl. Acad. Sci. USA* 94:2007-2012 (1997)). Brains were removed 5 hours following trauma or sham operation, and each hemisphere without 2 mm from frontal and occipital poles were homogenized. Sham-operated mice were craniectomized but not traumatized.

20 **Figure 7A-7C** Total lesion volume after traumatic brain injury in NSE-M17Z transgenic mice or corresponding wild-type littermates (Figure 7A) or in C57BL/6 mice (Figure 7B and 7C). Lesion size was assessed 24 hours following weight drop impact to the right hemisphere. zVAD-fmk (480 ng) was injected i.c.v. either 1 hour before trauma (B) or 1 hour after trauma (C). Total lesion volume is calculated from lesion areas determined in each of 5 coronal sections (2 mm) from anterior (2 mm behind frontal pole) to posterior (10 mm). Total lesion volume was decreased in NSE-M17Z transgenic mice and after injection

25

of zVAD-fmk 1 hour before trauma. Although total lesion volume in animals injected with zVAD-fmk 1 hour after trauma was not significantly different, the size of lesion in the treated mice in the two most anterior sections (2-6 mm) was significantly reduced ($P < 0.05$; $n = 5$; data not shown) indicating a trend of a protective effect. Data are presented as mean \pm SEM ($n = 5-6$). * $P < 0.05$ vs. vehicle or, in case of transgenic mice, vs. wild-type littermates.

Figure 8. Trauma-induced increase in free radical production in M17Z and wild-type mouse brain homogenates. Results are shown as 3,4-DHBA (3,4-dihydroxybenzoic acid)/4-HBA (4-hydroxybenzoic acid) ratio and represent the change in free radical production when compared to ipsilateral hemispheres of sham-operated mice. Data are presented as mean \pm SEM ($n = 7$); * $P = 0.03$.

Detailed Description of Preferred Embodiments

In the description that follows, a variety of technical terms are used. Unless the context indicates otherwise, these terms shall have their ordinary well-recognized meaning in the art. In order to provide a clearer and more consistent understanding of the specification and claims, the following definitions are provided.

Italicized words such as "*ICE*" refer to the gene while non-italicized words such as "*ICE*" refer to the RNA or protein product encoded by the corresponding gene.

ALS or ALS-like Symptoms. As used herein, the terms "ALS or ALS-like symptoms" refers to asymmetric weakness in two or more limbs, progressing to complete paralysis. This may also be described as an age-dependent progressive motor weakness. Onset of the disease may be described by a significantly slower gait than corresponding control subjects.

Active Site As used herein, "active site" refers to the catalytic site of the ICE protein. In human ICE, the active site comprises at least amino acids 283-287, while in the murine ICE this comprises at least amino acids 282-286. The active site contains the consensus amino acid sequence QACRG (SEQ ID No. 12).

Apoptosis. As used herein, "apoptosis" refers to the process by which organisms eliminate unwanted cells. The process is regulated by a cellular program. Apoptosis may eliminate cells during normal development, aging, tissue homeostasis or following imposition of an external stress such as hypoxia or trophic factor deprivation or during a disease state such as in ALS.

Central Nervous System Damage. As used herein, "central nervous system damage refers to any injury to the central nervous system that results in programmed cell death or apoptosis of neurons. Specific examples of such damage is that which results from ALS, traumatic brain injury (TBI), Alzheimer's disease, stroke and spinal cord injury. Such examples, however, are not meant to be limiting and also include other central nervous system damage recognized by those of skill in the art to result from neuronal apoptosis.

Dominant Negative Inhibitor. As used herein, "dominant negative inhibitor" refers to a mutated version of the wild type protein, that when expressed in cells can inhibit the activity of the endogenous protein.

Expression vector. As used herein, an "expression vector" is a vector comprising a structural gene operably linked to an expression control sequence so that the structural gene can be expressed when the expression vector is transformed into an appropriate host cell. Two DNA sequences are said to be "operably linked" if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region sequence to direct the transcription of the desired sequence, or (3) interfere with the ability of the desired sequence to be transcribed by the promoter region sequence. Thus, a promoter region would be

operably linked to a desired DNA sequence if the promoter were capable of effecting transcription of that DNA sequence.

5 **Gene therapy.** As used herein, "gene therapy" means, *inter alia*, the ability to ameliorate or eliminate a genetic defect by altering the gene of interest or the product expressed by the gene of interest, by altering the genotype of the cell or organism of interest. For example, this may be accomplished by replacing the gene with a mutated gene, knocking out the gene of interest or inserting a different gene that produces a product that inhibits or stimulates the gene of interest or using other methods known to those of skill in the art. The
10 manipulation of the genetic material may be accomplished either *in vivo* or *ex vivo*. These examples are not to be construed as limiting the different ways in which the gene therapy may be effected.

15 **ICE pathway.** As used herein, "ICE pathway" refers to the pathway by which interleukin-1 β converting enzyme is activated and converts the pro-IL β to mature IL- β eventually resulting in programmed cell death.

Modulating programmed cell death. As used herein, "modulating programmed cell death" should be understood to mean that one either increases or decreases cell death depending upon the desired end result.

20 **Mutated gene.** As used herein, "mutated gene" refers to a gene containing an insertion, substitution, or deletion resulting in the loss of substantially all of the biological activity associated with the gene. For example, a mutated *ICE* gene may either not express the protein of interest or if the substitution is minimal may express the protein of interest, but the protein may have a loss of biological activity. The term "biological activity" is readily understood by those of skill in
25 the art. For example, the biological activity of an enzyme relates to the ability of the enzyme to act on its appropriate substrate and effect catalysis of the reaction converting the substrate to the appropriate product. Alternatively, biological activity of a growth factor could be that activity which stimulates a target cell to divide or express a specific protein.

For the purposes of exemplification, a specific example of a gene encoding a mutation in a murine ICE protein (C284G) is presented in the specification. However, similar mutations (e.g. in the active site of ICE) in genes encoding corresponding ICE proteins from other species such as in human ICE are within the scope of this invention.

Resistant to or attenuated. As used herein "resistant to" or attenuated" means that an animal exposed to a certain treatment shows a greater degree of survivability, will live longer than the corresponding control (i.e. the treatment results in decreased lethality from the disease or condition than what is observed in the corresponding control) or will show an improvement in the disease symptoms. This does not necessarily mean that all animals will survive the treatment or that the animals will recover from the disease..

Targeting vector. As used herein "a targeting vector" is a vector comprising sequences that can be inserted into a gene to be disrupted, e.g., by homologous recombination. Therefore, a targeting vector may contain sequences homologous to the gene to be disrupted.

Transgenic. As used herein, a "transgenic organism" is an organism containing a defined change to its germ line, wherein the change is not ordinarily found in wild-type organisms. This change can be passed on to the organism's progeny and therefore the progeny are also transgenic animals. The change to the organism's germ line can be an insertion, a substitution, or a deletion in the gene of interest. The term "transgenic" also encompasses organisms containing modifications to their existing genes and organisms modified to contain exogenous genes introduced into their germ line. Thus, the term also "transgenic" also encompasses organisms where a gene has been eliminated, modified or disrupted so as to result in the elimination of a phenotypic characteristic associated with the disrupted gene (e.g. "knock-out animals"). This invention relates to non-human transgenic animals comprising a mutant *ICE* gene and a mutant *SOD* gene.

Vector. As used herein, a "vector" is a plasmid, phage, or other DNA sequence, which provides an appropriate nucleic acid environment for a transfer of a gene of interest into a host cell. Cloning vectors will ordinarily replicate autonomously in eukaryotic hosts. The cloning vector may be further characterized in terms of endonuclease restriction sites where the vector may be cut in a determinable fashion. The vector may also comprise a marker suitable for use in identifying cells transformed with the cloning vector. For example, markers can be antibiotic resistance genes.

Gene Therapy

A patient (human or non-human) suffering from ALS symptoms may be treated by gene therapy. By undertaking this approach, there should be an attenuation of the ALS symptoms. Gene therapy approaches have proven effective or to have promise in the treatment of certain forms of human hemophilia (Bontempo, F.A., *et al.*, *Blood* 69:1721-1724 (1987); Palmer, T.D., *et al.*, *Blood* 73:438-445 (1989); Axelrod, J.H., *et al.*, *Proc. Natl. Acad. Sci. USA* 87:5173-5177 (1990); Armentano, D., *et al.*, *Proc. Natl. Acad. Sci. USA* 87:6141-6145 (1990)), as well as in the treatment of certain other mammalian diseases such as cystic fibrosis (Drumm, M.L., *et al.*, *Cell* 62:1227-1233 (1990); Gregory, R.J., *et al.*, *Nature* 347:358-363 (1990); Rich, D.P., *et al.*, *Nature* 347:358-363 (1990)), Gaucher disease (Sorge, J., *et al.*, *Proc. Natl. Acad. Sci. USA* 84:906-909 (1987); Fink, J.K., *et al.*, *Proc. Natl. Acad. Sci. USA* 87:2334-2338 (1990)), muscular dystrophy (Partridge, T.A., *et al.*, *Nature* 337:176-179 (1989); Law, P.K., *et al.*, *Lancet* 336:114-115 (1990); Morgan, J.E., *et al.*, *J. Cell Biol.* 111:2437-2449 (1990)), and metastatic melanoma (Rosenberg, S.A., *et al.*, *Science* 233:1318-1321 (1986); Rosenberg, S.A., *et al.*, *N. Eng. J. Med.* 319:1676-1680 (1988); Rosenberg, S.A., *et al.*, *N. Eng. J. Med.* 323:570-578 (1990)).

In a preferred approach, a polynucleotide having the nucleotide sequence depicted in Figure 2D (SEQ ID NO:3), that of the cDNA clone deposited as

pJ655, ATCC Accession No. 209077 or a degenerate variant of the sequence, a nucleic acid molecule encoding an ICE inhibitor, or a nucleic acid molecule complementary to said inhibitor, or an anti-sense sequence for the *ICE* gene may be incorporated into a vector suitable for introducing the nucleic acid molecule into cells of the mammal to be treated, to form a transfection vector.

Knowing the amino acid sequence of an ICE inhibitor, one of skill in the art may readily determine the corresponding nucleic acid sequence based on the the triplet codons for each amino acid. Conversely, knowing the DNA sequence one may readily determine the derived amino acid sequence. Furthermore, knowing the triplet codon for an amino acid, one can also readily determine degenerate variants of that triplet codon such that they still encode the same amino acid sequence.

Suitable vectors for this purpose include retroviruses and adenoviruses. Alternatively, the nucleic acid molecules of the invention may be complexed into a molecular conjugate with a virus (e.g., an adenovirus) or with viral components (e.g., viral capsid proteins).

Techniques for the formation of such vectors comprising the inhibitor nucleic acid molecule or an anti-sense sequence to the *ICE* gene are well-known in the art, and are generally described in "Working Toward Human Gene Therapy," Chapter 28 in *Recombinant DNA, 2nd Ed.*, Watson, J.D. *et al.*, eds., New York: Scientific American Books, pp. 567-581 (1992). In addition, general methods for construction of gene therapy vectors and the introduction thereof into affected animals for therapeutic purposes may be found in the above-referenced publications, the disclosures of which are specifically incorporated herein by reference in their entirety.

In one such general method, vectors comprising the isolated mutant *ICE* gene are directly introduced into the cells or tissues of the affected individual, preferably by injection, inhalation, ingestion or introduction into a mucous membrane via solution; such an approach is generally referred to as "*in vivo*" gene therapy. Alternatively, cells or tissues, e.g., hematopoietic cells from bone

marrow, may be removed from the affected animal and placed into culture according to methods that are well-known to one of ordinary skill in the art; the vectors comprising the polynucleotides may then be introduced into these cells or tissues by any of the methods described generally above for introducing
5 isolated polynucleotides into a cell or tissue, and, after a sufficient amount of time to allow incorporation of the polynucleotides, the cells or tissues may then be re-inserted into the affected animal or a second animal in need of treatment. Since the introduction of the DNA of interest is performed outside of the body of the affected animal, this approach is generally referred to as "*ex vivo*" gene therapy.

10 For both *in vivo* and *ex vivo* gene therapy, the polynucleotides of the invention may alternatively be operatively linked to a regulatory DNA sequence, which may be a heterologous regulatory DNA sequence, to form a genetic construct as described above. This genetic construct may then be inserted into a vector, which is then directly introduced into the affected animal in an *in vivo*
15 gene therapy approach, or into the cells or tissues of the affected animal in an *ex vivo* approach. In another preferred embodiment, the genetic construct may be introduced into the cells or tissues of the animal, either *in vivo* or *ex vivo*, in a molecular conjugate with a virus (*e.g.*, an adenovirus) or viral components (*e.g.*, viral capsid proteins).

20 The above approaches result in (a) homologous recombination between the nucleic acid molecule and the defective gene in the cells of the affected animal; (b) random insertion of the gene into the host cell genome; or (c) incorporation of the gene into the nucleus of the cells where it may exist as an extrachromosomal genetic element. General descriptions of such methods and
25 approaches to gene therapy may be found, for example, in U.S. Patent No. 5,578,461; WO 94/12650; and WO 93/09222.

Alternatively, transfected host cells, which may be homologous or heterologous, may be encapsulated within a semi-permeable barrier device and implanted into the affected animal, allowing passage of for example, the ICE

inhibitor into the tissues and circulation of the animal but preventing contact between the animal's immune system and the transfected cells (see WO 93/09222).

Vectors

5 A variety of vectors have been developed for gene delivery to the nervous system and to brain tumors. These vectors derive from herpes simplex virus type 1 (HSV-1), adenovirus, adeno-associated virus (AAV) and retrovirus constructs (for review see Friedmann, T., *Trends Genet* 10:210-214 (1994); Jolly, D., *Cancer Gene Therapy* 1 (1994); Mulligan, R.C., *Science* 260:926-932
10 (1993); Smith, F. *et al.*, *Rest. Neurol. Neurosci.* 8:21-34 (1995)). Vectors based on HSV-1, including both recombinant virus vectors and amplicon vectors, as well as adenovirus vectors can assume an extrachromosomal state in the cell nucleus and mediate limited, long term gene expression in postmitotic cells, but not in mitotic cells. HSV-1 amplicon vectors can be grown to relatively high
15 titers (10^7 transducing units/ml) and have the capacity to accommodate large fragments of foreign DNA (at least 15 kb, with 10 concatemeric copies per virion). AAV vectors (rAAV), available in comparable titers to amplicon vectors, can deliver genes (<4.5 kb) to postmitotic, as well as mitotic cells in combination with adenovirus or herpes virus as helper virus. Long term transgene expression
20 is achieved by replication and formation of "episomal" elements and/or through integration into the host cell genome at random or specific sites (for review see Samulski, R.J., *Current Opinion in Genetics and Development* 3:74-80 (1993); Muzyczka, N., *Curr. Top. Microbiol. Immunol.* 158:97-129 (1992)). HSV, adenovirus and rAAV vectors are all packaged in stable particles. Retrovirus
25 vectors can accommodate 7-8 kb of foreign DNA and integrate into the host cell genome, but only in mitotic cells, and particles are relatively unstable with low titers. Recent studies have demonstrated that elements from different viruses can be combined to increase the delivery capacity of vectors. For example, incorporation of elements of the HIV virion, including the matrix protein and

integrates, into retrovirus vectors allows transgene cassettes to enter the nucleus of non-mitotic, as well as mitotic cells and potentially to integrate into the genome of these cells (Naldini, L. *et al.*, *Science* 272:263-267 (1996)); and inclusion of the vesicular stomatitis virus envelope glycoprotein (VSV-G) increases stability of retrovirus particles (Emi, N. *et al.*, *J. Virol.* 65:1202-1207 (1991)).

HSV-1 is a double-stranded DNA virus which is replicated and transcribed in the nucleus of the cell. HSV-1 has both a lytic and a latent cycle. HSV-1 has a wide host range, and infects many cell types in mammals and birds (including chicken, rat, mice monkey, and human) Spear et al., *DNA Tumor Viruses*, J. Tooze, Ed. (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1981) pp. 615-746. HSV-1 can lytically infect a wide variety of cells including neurons, fibroblasts and macrophages. In addition, HSV-1 infects post-mitotic neurons in adult animals and can be maintained indefinitely in a latent state. Stevens, *Current Topics in Microbiology and Immunology* 70: 31(1975). Latent HSV-1 is capable of expressing genes.

AAV also has a broad host range and most human cells are thought to be infectable. The host range for integration is believed to be equally broad. AAV is a single stranded DNA parvovirus endogenous to the human population, making it a suitable gene therapy vector candidate. AAV is not associated with any disease, therefore making it safe for gene transfer applications (Cukor et al., *The Parvoviruses*, Ed. K. I. Berns, Plenum, N.Y., (1984) pp. 33-36; Ostrove et al., *Virology* 113: 521 (1981)). AAV integrates into the host genome upon infection so that transgenes can be expressed indefinitely (Kotin et al., *Proc. Natl. Acad. Sci. USA* 87: 221 (1990); Samulski et al., *Embo J.* 10: 3941 (1991)). Integration of AAV into the cellular genome is independent of cell replication which is particularly important since AAV can thus transfer genes into quiescent cells (Lebkowski et al., *Mol. Cell. Biol.* 8:3988 (1988)).

Both HSV and AAV can deliver genes to dividing and non-dividing cells. In general, HSV virions are considered more highly infectious than AAV virions,

with a ratio of virus particles: infectious units in the range of 10 for HSV (Browne, H. *et al.*, *J. Virol.* 70:4311-4316 (1996)) and up to thousands for AAV (Snyder, R.O. *et al.*, In Current Protocols in Human Genetics, Eds. Dracopoli, N. *et al.*, John Wiley and Sons: New York (1996), pp. 1-24), and both having a
5 broad species range. Still, each virion has specific tropisms which will affect the efficiency of infection of specific cell types. The recent identification of a membrane receptor for HSV-1 which is a member of the tumor necrosis factor alpha family (Montgomery, R.I. *et al.*, 21st Herpes Virus Workshop Abstract #167 (1996)) indicates that the distribution of this receptor will affect the relative
10 infectability of cells, albeit most mammalian cell types appear to be infectable with HSV-1. AAV also has a very wide host and cell type range. The cellular receptor for AAV is not known, but a 150 kDA glycoprotein has been described whose presence in cultured cells correlates with their ability to bind AAV (Mizukami, H. *et al.*, *Virology* 217:124-130 (1996)).

15 *Protease inhibitors*

ICE has been identified as a cysteine protease and peptide aldehyde inhibitors of ICE have been described (Thornberry *et al.*, *Nature* 356:768-774 (1992). Additionally, other peptide inhibitors of the ICE family delay motor neuron death *in vitro* and *in vivo* (Milligan *et al.*, *Neuron* 15:385-393 (1995);
20 Hara *et al.*, *Proc. Natl. Acad. Sci. USA* 94:2007-2012 (1997)). These inhibitors have been shown to arrest programmed cell death of motoneurons *in vivo* and *in vitro* during the period of naturally occurring cell death accompanying development (Milligan *et al.*, *Neuron* 15:385-393 (1995)). The inhibitors effects have also been shown to reduce ischemic and excitotoxic neuronal damage during
25 reperfusion following filamentous middle cerebral occlusion (Hara *et al.*, *Proc. Natl. Acad. Sci. USA* 94:2007-2012 (1997)). The peptide inhibitors used in these two different experiments included N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD.FMK), acetyl-Tyr-Val-Ala-Asp-chloromethylketone

(Ac-YVAD.CMK), N-benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone (z-DEVD.FMK) and Ac-YVAD-CHO. z-VAD-FMK (SEQ ID No. 29) and z-DEVD.FMK - Enzyme Systems Products - Dublin, CA; Ac-YVAD-CMK and YVAD-CHO - Bachem Biosciences - King of Prussia, PA).

5 Thus, the ICE protease family plays an important role in mammalian neuronal apoptosis.

10 The claimed invention provides a method of treating ALS symptoms and further provides a transgenic mouse model to study the disease. The transgenic mouse model of the invention comprises a mouse with attenuated ALS symptoms that provides, *inter alia*, a model and/or test system for investigators to manipulate and better understand the mechanisms of apoptosis and ALS. In particular, a better understanding is gained concerning the role of the ICE gene and the ICE pathway. Such a model, allows the investigator to test various drugs where physiological responses are altered in the mouse, and thereby determine
15 more effective therapies to treat the underlying mechanism of ALS. Thus, the transgenic animals of this invention are also useful as animal models to study apoptosis and ALS

20 Therefore, invention also provides, a method of screening compounds, comprising: providing the compound to a transgenic non-human animal having a mutant SOD-1 gene and a mutant *ICE* gene and then determining the effect of the compound on apoptosis of said animal; and correlating the effect of the compound with increases or decreases in apoptosis.

25 The compounds to be tested can be administered to the animal having ALS and a mutant *ICE* gene in a variety of ways well known to one of ordinary skill in the art. For example, the compound can be administered by parenteral injection, such as subcutaneous, intramuscular, or intra-abdominal injection,

infusion, ingestion, suppository administration, and skin-patch application. Moreover, the compound can be provided in a pharmaceutically acceptable carrier. See "Remington's Pharmaceutical Sciences" (1990). The effect of the compound on apoptosis and ALS can be determined using methods well known to one of ordinary skill in the art.

These aspects of the invention (i.e. those relating to the testing of compounds affecting apoptosis or ALS) are useful to screen compounds from a variety of sources. Examples of compounds that can be screened using the method of the invention include but are not limited to rationally designed and synthetic molecules, plant extracts, animal extracts, inorganic compounds, mixtures, and solutions, as well as homogeneous molecular or elemental samples. Establishing that a compound has an effect in the mutant animals has predictive value relating to that compound's effect in other animals, including humans. Such predictive values provide for initial screening of therapeutically valuable drugs.

The invention, therefore, provides a method of screening compounds, comprising: providing a transgenic non-human animal demonstrating ALS symptom having mutant *SOD* and *ICE* genes, said animal exhibiting the an attenuated form of ALS, administering a compound to be tested to the transgenic animal; determining the effect of the compound on the properties of interest in said animal; and correlating the effect of the compound on the mouse with the effect of said compound in a control animal.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration. The general description of the invention, as well as the following examples, are not intended to be limiting of the present invention.

Examples

It has previously been demonstrated that binding of endogenously produced mature IL-1 β to its type-1 receptor plays an important role in apoptosis (Friedlander *et al.*, *J. Exp. Med.* 184:717-724 (1996)). It has also been shown that replacing the cysteine in the active site of ICE with a glycine in the mouse (C284G) obliterates its ability to mediate cell death (Miura *et al.*, *Cell* 75:653-660 (1993)). The residue in the active site is required for the IL-1 β convertase and the autoprocessing activity of ICE (Gu *et al.*, *EMBO J.* 14:1923-1931 (1995)).

Example I

Mutant ICE Protects Dorsal Root Ganglion (DRG) Neurons

Survival of DRG neurons in culture requires the presence of trophic factors which include nerve growth factor and serum. In the absence of trophic factor support, DRG neurons undergo apoptosis (Davies *et al.*, *Development* 101:185-208 (1987)). To determine whether a mutant ICE can inhibit DRG neuronal death induced by trophic factor deprivation, primary cultures of chicken embryonic DRG neurons were microinjected with a construct of the fused mutant ICE^{C284G}-lacZ gene under the control of the β -actin promoter (β -actin-M17Z).

Methods

Screening of cDNA Library

Standard techniques of molecular cloning were used as described (Sambrook *et al.* *Molecular Cloning: A Laboratory Manual, Second Ed.*, Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press (1989)), unless otherwise indicated. A human ICE cDNA was obtained by polymerase chain reaction (PCR) using the human ICE sequence (Thornberry *et al.*, *Nature* 356, 768-774 (1992)). This cDNA was used as a probe to screen a mouse thymus

cDNA library (Stratagene, LaJolla, California). The filters were hybridized in 5x SSPE, 20% formamide, 0.02% Ficoll, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 1% SDS at 40°C for 2 days and washed in 1x SSPE and 0.5% SDS for 20 min, twice at room temperature and twice at 45°C.

5 Plasmid Construction

pJ415 was constructed by first inserting a 5'400 bp BglII-BamHI *crmA* fragment into the BamHI site of the pBabe/puro vector and then inserting the remaining 1 kb BamHI *crmA* fragment into the 3' BamHI site in a sense direction. pJ436 was constructed by inserting an EcoRI-Sall *bcl-2* fragment into the EcoRI-Sall sites of the pBabe/puro vector (Morgenstern *et al.*, *Nucl. Acids Res.* 18:3587-3596 (1990)). To construct fusion genes, the E.coli β -galactosidase gene was taken from the plasmid 407-794.Z (Picard *et al.*, *EMBO J.* 6:3333-3340 (1987)) by digestion with BamHI and cloned into pBlue-script vector (BSlacZ). Various 5' deletion fragments of mICE (pJ348) were obtained by PCR. PCR was performed by using synthetic primers (M10 and T3 primer for the whole mICE construct, M11 and T3 primer for the P20/P10 construct, M11 and M13 for the P20 construct, M12 and T3 primer for the P10 construct) and pJ348 as a template. The primer sequences were as follows:

M10 - AAGTCGACGCCATGGCTGACAAGATCCTGAGGG (SEQ ID No. 13),
 20 M11 - AAGTCGACGCCATGAACAAAGAAGATGGCACAT (SEQ ID No. 14);
 M12 - AAGTCGACGCCATGGGCATTAAGAAGGCCCAT (SEQ ID No.15);
 M13- TTCCCGGGTCATCTTCAAAAATTGCATCCG (SEQ ID No. 16). The amplified fragments were digested with Sall and SmaI and then cloned into Sall-SmaI sites of BSlacZ. BSced38Z was made by first inserting a SmaI-digested PCR product of *ced-3* cDNA (primers used were M18 and M19;

25 M18 - AACCCGGGAGGCCTCCATGATGCGTCAAGATAGAAG (SEQ ID No. 17); M19-AACCCGGGACGGCAGAGTTTCGTGCTTCCG) (SEQ ID No. 18) into BSlacZ. BSM 10Z (mICE-*IacZ* in pBluescript II vector), BSM11Z (P20/P10-*IacZ* in pBluescript II vector), BSM19Z (P20-*IacZ* cloned in

pBluescript II vector), BSM12Z (*P10-lacZ* cloned in pBluescript II vector), and BSced38Z (*ced-3-lacZ* cloned in pBluescript II vector) were digested with XhoI-NotI, blunt ended by Klenow fragment, and then cloned into p β actSTneoB (Miyawaki et al., 1990) (digested with Sall and blunt ended by Klenow fragment) individually, and the resulted plasmids were named p β actM10Z, p β actM11Z, p β actM12Z, and p β actced38Z, respectively. To mutate the glycine residue to a serine residue in the active domain of mICE, the PCR product of primers m8p/s (ATTCAGGCCTCCAGAGGAGAGAAAC) and mice 8 (GGCACGATTCTCAGCATAGGT), using pJ348 as a template, was digested with SphI and SmaI and then cloned into the SphI-SmaI sites of BSM10Z (pJ483). To mutate the cysteine residue to a glycine residue in the active domain of mICE, the PCR product of M10 and M15 (CAAGGCCTGCCTGAATAATGATCACCTT), using pJ348 as a template, was digested with Sall and StuI, then cloned into BSM10Z that was digested with SphI and blunt ended by T4 DNA polymerase, and then digested with Sall (BSM17Z). To mutate the glycine residue to a serine residue in the active domain of *ced-3*, the PCR products of the C-terminal portion of CED-3 (primers used were M19 and M20; M20, GCAGGCCTGTCGATCGGAACGTCGTGACAATGGATT) and the N-terminal portion of CED-3 (primers used were M18 and M21; M21, ACAGGCCTGCACAAAAACGATTTT) were digested with StuI and SmaI and then cloned into SmaI site of BS β lacZ (BSced37Z). pJ483, BSM17Z, and BSced37Z were digested with XhoI and NotI, blunt ended by Klenow fragment, and then cloned into p β actSTneoB individually, and the resulted plasmids were named pJ485, p β actM17zs, and p β actced37Z, respectively. (Miura et al., *Cell* 75:653-660 (1993)).

The experiments were performed essentially as described by Gagliardini et al. (*Science* 263:826-828 (1994)). Primary cultures of chicken embryonic DRG neurons were isolated under sterile conditions from day 10 embryos

(Spafas, Preston, CT). DRG's were dissociated by incubating in trypsin for 15 minutes at 37°C and trituration. Dissociated neurons were plated on poly-L-lysine (Sigma, 30 mg/ml for 1 hour) and laminin (Sigma, 20 mg/ml for 2 hours) coated chamber slides. DRG neurons were cultured for 2 days in F12 medium (Gibco) containing 10% fetal bovine serum (Hyclone), penicillin (100 U/ml, Gibco), streptomycin (100 mg/ml, Sigma), and 5 mM cytosine b-D-arabinose (Sigma), supplemented with NGF (10 ng/ml, Sigma).

Neuron injection was performed with an Eppendorf microinjector (model 5242), with glass micropipettes loaded with 1 mg/ml plasmid DNA in TE buffer and 5% rhodamine dye (rhodamine-isothiocyanate labeled dextran, 10 kDa; Molecular probes, Eugene, OR), dissolved in 0.2 M KCl. The construction of the fused mutant ICE (C284G)-lacZ plasmid (β -actin-M17Z) was described by Miura et al (*Cell* 75:653-660 (1993)). Three hours after injection, the NGF containing medium was replaced with NGF-free and serum-free medium in the presence of sufficient mouse monoclonal antibody against NGF (Boehringer Mannheim, Indianapolis, IN). The medium was changed daily. Live injected neurons were counted on days 0, 3, and 6.

Neurons were co-injected with rhodamine-isothiocyanate dextran as a marker and with Hoechst dye to determine neuronal nuclear morphology. Following trophic factor removal, control neurons microinjected with the b-actin-lacZ construct survived 22.5 and 6.0% after 3 and 6 days in culture, respectively. No significant difference was detected when compared to cells injected with dye alone. In contrast, neurons injected with b-actin-M17Z survived 85.0 and 81.0% after 3 and 6 days in culture, respectively. These results showed that the mutant ICE gene inhibits DRG neuronal cell death induced by trophic factor deprivation, suggesting that mutant ICE may be able to suppress the activities of wild type ICE or ICE-like proteases.

Example 2

Generation of Transgenic Mice Expressing the Mutant ICE (C284G) Protein

5 To determine whether the mutant *ICE* gene can also act as an inhibitor of apoptosis *in vivo*, and to further evaluate its mechanism of action, transgenic mouse lines expressing the fused mutant ICE^{C284G}-lacZ gene under the control of the neuron specific enolase promoter (NSE-M17Z) were established.

10 pNSE-M17Z-lacZ construct was made by digesting pNSE-lacZ with Sall and ClaI which removed a 0.8 kb Sall/ClaI fragment. The Sall/ClaI digested pNSE-lacZ vector was ligated with a 2kb sall/ClaI insert from BSM17Z which contains the mutant ICE (C284G) and the part of lacZ which was removed in the Sall/ClaI digest of the pNSE/lacZ vector. The resulting construct was named pJ655. To generate transgenic mice, pJ655 was linearized by XmnI digestion and gel purified. Fourteen transgenic mice lines were generated by DNX (Princeton, 15 NJ). Founder mice were SV-129/C57BL/6 hybrid. Initially 5 lines were selected (7506, 7512, 7516, 7538, and 7539) based on highest DNA copy number in the genome.

Tail DNA was isolated and genotyping was performed using the following PCR primers targeted to the *Ice/lacZ* fusion (M17Z-F: 20 5'TGCCCAAGCTTGAAAGACAAGCCC3' (SEQ ID No. 24), lacZ-R: 5'CTGGCGAAAGGGGGATGTGCTG3') (SEQ ID No. 25). X-gal staining was performed by removing the vertebral column and sectioning it in a sagittal plane. Tissue was fixed for 5 minutes on ice (0.2% glutaraldehyde in 0.1M phosphate buffer, 2% formaldehyde, 5mM EGTA pH7.3, 2mM₂) followed by three 30 25 minute washes at 4°C (0.1M phosphate buffer pH7.3, 2mM MgCl₂, 0.1% sodium deoxycholate, 0.2% NP40). The tissue was then stained overnight with X-gal at 37°C (rinse solution with 1 mg/ml X-Gal in DMSO, 5mM K ferrocyanide, 5mM

K ferricyanide), and then sectioned in a cryostat (40 μ m). Photomicrographs were taken in a light microscope (100x) under oil immersion.

Transgenic mice expressing either the lacZ or bcl-2 genes under control of the NSE promoter have been well characterized, and transgene expression has been detected throughout the nervous system (Martinou *et al.*, *Neuron* 13:1017-1030 (1994); Forss-Petter *et al.*, *Neuron* 5:187-197 (1990); Farlie *et al.*, *Proc. Natl. Acad. Sci. USA* 92:4397-4401 (1994)). PCR was used for genotyping the NSE-M17Z transgenic mice, and protein expression was detected by X-gal staining. Founder mice from five different lines were crossed with C57BL/6 mice. The expression of NSE-M17Z was detected in the first and second generation offsprings which were used in some of the experiments described below.

Example 3

Mutant ICE Acts In Vivo as a Dominant Negative Inhibitor of ICE.

It was next evaluated whether the mutant ICE^{cm40} may act as a dominant negative ICE inhibitor. Ice knockout mice were almost completely defective in processing pro-IL-1 β and ICE is the only protease identified so far that can process pro-IL-1 β (Kuida *et al.*, *Science* 267:2000-20002 (1995); Li *et al.*, *Cell* 80:401-411 (1995). If the mutant Ice transgenic mice have a defect in secreting mature IL-1b, this would provide strong evidence that mutant ICE^{cm40} can act as a dominant negative inhibitor of ICE.

Systemic injection of lipopolysaccharide (LPS) induces release of mature IL-1 β . ICE knockout mice generated by gene-targeting technique were unable to release mature IL-1 β upon LPS stimulation (Kuida *et al.*, *Science* 267:2000-20002 (1995); Li *et al.*, *Cell* 80:401-411 (1995). To determine if mutant ICE^{cm40}

transgenic mice are also defective in secreting mature IL-1 β , LPS was injected intraperitoneally into the mutant ICE^{cmg} transgenic mice and the levels of mature IL-1 β were determined in whole brain lysates using an ELISA kit that specifically detects mature IL-1 β . Following the systemic LPS challenge, whole brain lysates of mutant ICE^{cmg} transgenic mice contained 74.7% less mature IL-1 β as compared to that of LPS-injected wild type mice. In control wild type mice injected intraperitoneally with PBS, there was low but detectable levels of mature IL-1 β in the brain (4.0 pg/g brain) whereas this cytokine was undetectable in the brain lysate of PBS (phosphate buffered saline) injected mutant ICE^{cmg} mice (Figure 4). Thus, mutant ICE^{cmg} can act as an effective inhibitor of pro-IL-1 β processing, strongly suggesting that mutant ICE^{cmg} is a dominant negative inhibitor of ICE itself.

Example 4
NSE-M17Z Transgenic Mice are Resistant to
Cerebral Ischemic Injury

To determine if ICE plays a role in apoptosis induced by ischemic insult and if the mutant ICE^{cmg} may act to reduce ischemic brain injury, it was investigated whether the mutant ICE^{cmg} transgenic mice were protected in a mouse focal cerebral ischemia model where apoptotic cell death has been reported (Li *et al.*, *Mol. Brain Res.* 28:164-168 (1995). Permanent middle cerebral artery (MCA) occlusion was performed in 14 wild-type and 11 transgenic mice, progeny of 5 different founder mice (7509, 7512, 7516, 7538, and 7539).

Experiments were done as described by Hara *et al.* (*J. Cereb. Blood Flow Metab.* 16:605-611 (1996), except that the occlusion of the MCA was continuous for 24 hours. Neurological grading was as follows: 0=no neurological deficits; 1=failure to extend the right forepaw; 2=circling to the contralateral side; 3=loss of walking or righting reflex. All experiments were done in a double blinded fashion. Values shown as mean \pm s.e.m.

Mice were scored neurologically 30 minutes and 24 hours following the occlusion. In the initial 30 minute evaluation, there was no significant difference in the neurological score. During the ensuing 24 hours, however, wild-type mice remained impaired, whereas transgenic mice improved neurologically (Figure 3A).
5 Mice were sacrificed at 24 hours, and infarct volume was quantified with 4% 2, 3, 5-triphenyltetrazolium chloride (TTC). Infarct volume was significantly smaller in NSE-M17Z [$66 \pm 11 \text{ mm}^3$ ($n=11$)] when compared to the wild-type litter-mate mice [$125 \pm 5 \text{ mm}^3$ ($n=14$)] (Figure 3B). Physiologic parameters were recorded in a separate set of transgenic and wild-type mice. Blood pressure,
10 arterial blood gases (PO_2 , PCO_2 , and blood pH), regional cerebral blood flow, and body temperature did not significantly differ in the two sets of mice, before and throughout thirty minutes of ischemia (Figure 3C). Thus, expression of mutant $\text{ICE}^{\text{C240Q}}$, a dominant negative inhibitor of ICE, protects neurons from ischemic insult.

15 *Summary of Examples 1-4*

Mutant $\text{ICE}^{\text{C240Q}}$ inhibits apoptosis in two different species (chicken and mouse) and under the control of two different promoters (β -actin and NSE). Evidence has been presented that mutant $\text{ICE}^{\text{C240Q}}$ acts as a dominant negative inhibitor of ICE by inhibiting processing of pro-IL-1 β . X-ray crystallography analysis showed that
20 ICE exists as a dimer of two p20 and two p10 subunits processed from two p45 precursor molecules (Wilson *et al.*, *Nature* 370:270-275 (1994)). Expression of catalytically inactive mutant of ICE may result in formation of inactive dimers which will inhibit endogenous wild type ICE function.

Since DRG neurons from Ice knockout mice were protected from trophic
25 factor deprivation-induced apoptosis as were DRG neurons from NSE-M17Z transgenic mice, it is believed that, at least in DRG neurons, the mutant $\text{ICE}^{\text{C240Q}}$ prevented neuronal cell death by inhibiting ICE activity. This is consistent with previous experiments demonstrating that the addition of the IL-1 receptor antagonist, a naturally existing IL-1 β antagonist, inhibited mouse DRG neuronal

cell death induced by trophic factor deprivation, suggesting that release of mature IL-1 β processed by ICE plays an active role in apoptosis induced by trophic factor deprivation (Friedlander *et al.*, *J. Exp. Med.* 184:717-724 (1996)).

The role of ICE-like proteases in apoptosis induced by cerebral ischemia in a mouse permanent focal stroke model was determined. NSE-M17Z transgenic mice suffered less tissue injury and less behavioral changes as a result of ischemic injury as compared to the wild type mice. Although it cannot be ruled out that the reduction of neuronal damage by mutant ICE was due to its ability to inhibit other members of the Ice family, evidence suggests that ICE itself plays an important role in cerebral ischemia-induced cell death.

Elevated levels of IL-1 β are detected following cerebral ischemia (Lui *et al.*, *Stroke* 24:1746-1751 (1993)). In addition, intraventricular administration of the IL-1 receptor antagonist decreases infarct size following permanent middle cerebral artery (MCA) occlusion (Relton *et al.*, *Brain Res. Bull.* 29:243-246 (1992)). It has also demonstrated that endogenously produced mature IL-1b plays an important role in hypoxia-mediated apoptosis *in vitro* (Friedlander *et al.*, *J. Exp. Med.* 184:717-724 (1996)). These results suggest the involvement of ICE and of mature IL-1b receptor binding in the mechanism of ischemia-induced cell death. The results further corroborate the notion that ICE plays an important role in apoptosis induced by ischemic injury.

Following exposure to certain death stimuli, the ICE cell death cascade is activated. As demonstrated apoptosis may be inhibited by blocking the ICE cell death cascade, either the activation of pro-ICE, the function of active ICE, or the product of ICE activity which is mature IL-1 β (Gagliardini *et al.*, *Science* 263:826-828 (1994); Friedlander *et al.*, *J. Exp. Med.* 184:717-724 (1996) Boudreau *et al.*, *Science* 267:891-893 (1995)). It cannot be ruled out, however, that mutant ICE^{C284G} may also cross-inhibit other cell death gene products, since subunits of different ICE family members sharing significant sequence homology may bind to each other forming hetero-oligomers (Gu *et al.*, *EMBO J* 14:1923-1931 (1995)). Since embryonic development of the mutant ICE^{C284G} and of Ice

knockout mice are normal and no significant defect in embryonic apoptosis was uncovered (Kuida *et al.*, *Science* 267:2000-2002 (1995); Li *et al.*, *Cell* 80:401-411 (1995)), a nonredundant function of ICE in developmental apoptosis has been ruled out. It has been demonstrated here that transgenic mice expressing a dominant negative mutant ICE are significantly protected from neuronal cell death induced by ischemic insult, suggesting that ICE may play an important role in pathological cell death. Our results suggest that ischemic-induced injury, and possibly other disorders featuring apoptosis, can be treated with inhibitors aimed at modulating the activity of the ICE protease family in order to reduce tissue injury and preserve brain function.

Example 5 *Inhibition of ICE Activity In vivo*

To determine if inhibition of ICE activity in vivo might halt the progression of the ALS-like syndrome in the mutant SOD-1 mice, 5 female NSE-M17Z mice from one transgenic founder mouse (Friedlander *et al.*, *J. Exp. Med.* 185:933-940 (1997)) having a mutant ICE gene (as described above) were crossed with one mutant SOD(G93R) male mouse (Gurney *et al.*, *Science* 264:1772-1775 (1994)). The genotypes of the progeny from these crosses were determined for the carriers of the mutant ICE and mutant SOD transgenes by PCR. Litter mates of SOD(G93R) and SOD(G93R); mutant ICE were monitored for the times of disease onset and death.

The onset of the disease was scored as the appearance of significantly slower gait than that of Litter mates and/or limb paralysis. The end point was scored as death or when flipped on its side and is unable to get up in 30 sec. The scorers were completely unaware of the genotypes of the mice or their birthdates.

It was found that although the timing of the disease onset in the mutant SOD and mutant SOD/mutant ICE (M17Z) transgenics is not different, the double

transgenic mice survive significantly longer (27 days) than the mutant SOD mice alone (11.7 days) following the onset of the disease (Table 1). These results indicated that expression of the dominant negative inhibitor of ICE in neurons of

Table 1. Delay of mortality in SOD/G93R transgenic mice by expression of a dominant negative inhibitor of ICE

	Onset (days)	Length of Disease	Mortality
SOD mean (n=24)	237.7±4.4	11.7±1.6	249.3±3.8
SOD/M17Z mean (n=22)	243.0±5.6	27.0±3.5	270.0±7.0
Unpaired t test (two tailed)	p=0.464	*p=0.0002	*p=0.0115
* indicate significant difference			

mutant SOD mice is able to slow significantly the time of the symptomatic progression of this disease and delays mortality. These results suggest the involvement of the ICE-like proteases in the disease progression in this ALS mouse model and possible therapeutic value of ICE inhibitors in the treatment of ALS in humans.

Furthermore, based on these observations one may treat ALS patients using a recombinantly made ICE mutant protein. The mutant protein is obtained by using an appropriate expression vector followed by isolation of the protein, all of which uses methods readily known to those of skill in the art. expressing. The treatment comprises contacting the cells of a patient (human or non-human) in need of treatment for ALS or ALS-like symptoms with the recombinantly made mutant ICE protein. Such contact may be made either *in vivo* or *in vitro*.

Example 6

Screening of Compounds that Affect ALS using Transgenic Mice with a Mutant ICE Gene and SOD Gene

5 The transgenic mice comprising a mutant *ICE* gene and mutant *SOD* gene exhibit a delayed mortality and increased timecourse for ALS. This may be related to the inhibition of the *ICE* gene product, ICE-related proteases or the ICE cell death pathway. Using the transgenic mouse of the invention to screen compounds allows the pre-clinical determination of combinations of compounds which would be beneficial in treating ALS in affected individuals.

10 For example, with the effects of the ICE gene product blocked in the transgenic animal of the invention, a drug may further attenuate the ALS symptoms. In an animal without the mutant *ICE* gene, the effect of the drug of interest may not be determinable because any amelioration of symptoms it might produce are overcome by the effects of the ICE gene product. This problem should be minimized in a transgenic SOD mutant mouse that also expresses the mutant *ICE* gene.

15 Alternatively, if a drug potentiates the symptoms of ALS, its use may be contra-indicated for therapy. Thus, the mutant mice may be used for screening compounds for treating ALS and its related symptoms

20 Compounds to be screened for activity can be administered to the transgenic mice with the mutant *ICE/SOD* genes using pharmaceutically acceptable methods. See Remington's Pharmaceutical Sciences (1990). For example, the compound to be screened can be administered at various concentrations by parenteral injection, infusion, ingestion, and other suitable methods in admixture with a pharmaceutically acceptable carrier. The effect of various concentrations of the screened compound on increasing or decreasing the symptoms and mortality to ALS is measured. This is determined relative to control SOD mutant transgenic animals without the mutant *ICE* gene, transgenic animals that have not been administered the compound and wild-type animals without either the *SOD* or the

25

30 *ICE* mutant genes.

A significant delay in mortality or increase in the time in which the ALS symptoms are expressed in mice with the mutant *ICE/SOD* genes, following treatment with a screened compound may be indicative that the compound would exhibit beneficial effects in the treatment of ALS. Such a benefit may be obtained either alone or in combination with a compound that inhibits ICE activity.

Particularly preferred compounds for screening are those compounds known to inhibit activities of ICE *in vitro* or any other candidate for treating ALS.

Example 7

Treatment of ALS -Gene Therapy

A patient (human or non-human) with ALS symptoms is treated by gene therapy such that the effects of the ICE gene product are blocked. This may be accomplished by using the mutant *ICE* gene as described in Example 2. Alternatively, a human mutant *ICE* gene is used that contains a mutation in the active site of the ICE, e.g. the cysteine may be replaced with a glycine at amino acid 285, resulting in a C285G mutant rather than a C284G mutant (as in the mouse). The sequence of the human *ICE* gene can be obtained in the art (Thornberry, N.A., *Nature* 356:768-774 (1992)). Other mutations in the active site or elsewhere in the gene may also be appropriate. An appropriate vector such as adenovirus or herpes virus is chosen to infect the patient and the mutant gene is thereby directly introduced into the cells of the affected individual.

Alternatively, cells or tissues may be removed from the affected individual and placed into culture. The mutant *ICE* gene is then introduced into the cultured cells or tissues and then re-inserted into the patient.

Example 8

Treatment of ALS -Protease Inhibitors

A patient (human or non-human) with ALS symptoms is treated with protease inhibitors such that the effects of the ICE gene product are blocked and the ALS symptoms are attenuated. The protease inhibitors are selected from the group consisting of N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-

VAD.FMK), acetyl-Tyr-Val-Ala-Asp-chloromethylketone (YVAD.CMK), N-benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone (z-DEVD.FMK) and Ac-YVAD-CHO.

Example 9

5 *Post-traumatic Brain Injury and Oligonucleosomal DNA degradation*

Necrotic and apoptotic cell death both play a role mediating tissue injury following brain trauma. The Interleukin-1 β converting enzyme (ICE) is activated and oligonucleosomal DNA fragmentation is detected in traumatized brain tissue. Reduction of tissue injury and free radical production following brain trauma was
10 achieved in a transgenic mouse expressing a dominant negative inhibitor of ICE in the brain. Neuroprotection was also conferred by intracerebroventricular administration of the caspase inhibitor N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD-fmk). These results indicate that inhibition of ICE-like caspases reduces trauma-mediated brain tissue injury. In addition, *in vivo*
15 functional interaction between ICE-like caspases and free radical production pathways, implicating free radical production as a downstream mediator of the caspase cell death cascade has been demonstrated.

A transgenic mouse expressing a dominant negative inhibitor of ICE, which has the active site cysteine substituted for glycine (C285G), in neurons under the
20 control of the neuron specific enolase promotor (NSE-M17Z) was generated (Friedlander, R.M., *et al.*, *J. Exp. Med.* 185:933-940 (1997)). ICE was activated following cerebral ischemia, and expression of the M17Z transgene decreased ischemia-induced cerebral infarct, as well as mature IL-1 β production (Hara, H.,
et al., *Proc. Natl. Acad. Sci. USA* 94:2007-2012 (1997); Hara, H., *et al.*, *J. Cereb.*
25 *Blood Flow Metab.* 17:370-375 (1997); Friedlander, R.M., *et al.*, *J. Exp. Med.* 185:933-940 (1997)). Furthermore, synthetic peptide inhibitors of ICE-like caspases reduced infarct size following focal cerebral ischemia (Hara, H., *et al.*,
Proc. Natl. Acad. Sci. USA 94:2007-2012 (1997); Loddick, S.A., *et al.*,

NeuroReport 7:1465-1468 (1996)). ICE-mediated cell death also plays a role in the progression of amyotrophic lateral sclerosis (ALS) in the familial ALS transgenic mouse model (Friedlander, R.M., *et al.*, *Nature* 388:31 (1997)). The above evidence implicates ICE-like caspases as important mediators of cell death in a variety of neurological conditions.

Although apoptosis has been detected in experimental brain injury models (Colicos, M.A. & Dash, P.K., *Brain Res.* 739:120-131 (1996); Yakovlev, A.G. *et al.*, *J. Neurosci.* 17:7415-7424 (1997); Sinson, G., *et al.*, *J. Neurosurg.* 86:511-518 (1997)), this is the first study to specifically investigate the role of the ICE cell death pathway *in vivo* following cerebral trauma. Inhibition of this apoptotic pathway might attenuate traumatic cerebral injury. It is shown that ICE is activated in a traumatic brain injury model and that inhibition of the ICE cell death cascade, both by genetic and pharmacologic means reduces traumatic brain injury.

Methods

Traumatic brain injury.

Brain trauma experiments and lesion quantification were performed essentially as described (Chan, P.H., *et al.*, *Ann. NY Acad. Sci.* 738:93-103 (1994)). Spontaneously ventilating adult mice were initially anesthetized with halothane in 70% N₂O and 30% O₂, and fixed in a stereotactic frame. Before trauma, an atraumatic craniectomy was performed by removing the right parietal bone posterior to the coronal, lateral to the sagittal, and anterior to the lambdoid suture. Laterally, the craniectomy was extended to the insertion of the temporalis muscle. A piston which was 3 mm in diameter, and had an excursion of 3mm was then placed over the craniectomy defect. A 20 g weight was dropped inside a cylinder from a height of 150 mm onto the piston (final speed $v = 1.70$ m/s). Twenty-four hours after trauma, brains were removed and sectioned into five coronal (2mm) slices and stained with 2% 2,3,5-triphenyl tetrazolium chloride (TTC) as described for a weight drop trauma model (Chan, P.H., *et al.*, *Ann. NY Acad. Sci.* 738:93-103 (1994)). Surgical procedure as well as quantification of

lesion size was performed by an investigator naive to the animal identity. Lesion volume was calculated using an image analysis system (M4, Imaging Research, St. Catherines, Ontario, Canada) from the total lesion volume integrated from the volumes of each single section, after subtracting the volume of the deficient cortex as caused by piston penetration. The volume of the piston penetration was calculated as that of a cylinder ($\pi r^2 h = 3.14 \times 1.5^2 \times 3 \text{ mm}^3 = 21.2 \text{ mm}^3$), where r is the radius of the piston, and h the depth of penetration. The trauma protocol was approved by the IACUC. The NSE-M17Z and wild-type littermate mice were bred from C57BL/6 background, and the wild-type mice used for zVAD-fmk injection experiments were C57BL/6 (Taconic Farms, Germantown, NY). NSE-M17Z mice were genotyped as previously described (Friedlander, R.M., *et al.*, *J. Exp. Med.* 185:933-940 (1997)). zVAD-fmk (480 ng) or vehicle (DMSO 0.4%) was injected i.c.v. (2 μ l; bregma -0.9 mm lateral, -0.1 mm posterior, -3.1 mm deep) 1 hour before or 1 hour after trauma.

15 DNA fragmentation analysis.

DNA was end-labeled with [32 P]ddATP, electrophoresed on a 2% agarose gel and autoradiographed. For analysis of DNA damage, tissue samples corresponding to the striatum in slice 3 were obtained 24 hours after trauma. DNA was isolated using a kit (Puregene), digested with DNase-free RNase (Boehringer Mannheim) and extracted with phenol-chloroform. DNA was reprecipitated in ethanol, pelleted and resuspended. DNA concentration was quantified by absorbance at 260 nm. For visualization of damaged DNA, strand breaks were end-labeled with [32 P]ddATP (Tilly, J.L. & Hsueh, A.J.W., *J. Cell Physiol.* 154:519-526 (1993); MacManus, J.P., *et al.*, *Mol. Neurosci.* 5:493-496 (1995)). Three μ g of DNA were used for labeling, electrophoresed on a 2.0% agarose gel (agarose 3:1, Amresco), and detected by autoradiography.

Statistics.

Data are presented as mean \pm SEM. Statistical comparisons were made by

Student's t-test. For comparison of lesion size two-way ANOVA followed by Tukey posthoc tests was applied. $P < 0.05$ was considered statistically significant. For statistical evaluation by Student's t-test, data on OH production was log transformed.

5 *Results*

To determine whether apoptotic cell death develops as a consequence of direct impact brain injury, traumatized tissue was examined for the presence of oligonucleosomal DNA fragmentation. Oligonucleosomal DNA degradation was detected following experimental traumatic brain injury.

10 In the lesioned hemisphere, extensive DNA fragmentation was found 24 hours following trauma. DNA damage was not detected in brain tissue from sham-operated mice (Fig. 5). DNA fragments appeared on agarose gels as a ladder reflecting oligonucleosomal DNA fragmentation superimposed upon a smear reflecting random DNA degradation. Random DNA degradation results from
15 necrotic cell death, whereas oligonucleosomal DNA fragmentation occurs following apoptotic cell death. This result indicated that both necrotic as well as apoptotic cell death pathways are activated and likely play a role in experimental TBI.

Example 10

20 *ICE is activated following traumatic brain injury.*

Pro-IL- 1β processing requires functional ICE activity as demonstrated in ICE knock-out mice following lipopolysaccharide challenge (Li, P., *et al.*, *Cell* 80:401-411 (1995); Kuida, K., *et al.*, *Science* 267:2000-2003 (1995)). Therefore, detection of mature IL- 1β has been employed as direct evidence for ICE activation
25 (Miura, M., *et al.*, *Proc. Natl. Acad. Sci. USA* 92:8318-8322 (1995); Hara, H., *et al.*, *Proc. Natl. Acad. Sci. USA* 94:2007-2012 (1997); Hara, H., *et al.*, *J. Cereb. Blood Flow Metab.* 17:370-375 (1997); Troy, C.M., *et al.*, *Proc. Natl. Acad. Sci. USA* 93:5635-5640 (1996); Friedlander, R.M., *et al.*, *J. Exp. Med.* 185:933-940

(1997); Li, P., *et al.*, *Cell* 80:401-411 (1995); Kuida, K., *et al.*, *Science* 267:2000-2003 (1995)). In traumatized mice, brain tissue mature IL-1 β levels were significantly increased to 37.3 ± 5.2 pg/g brain tissue as compared with 16.6 ± 3.6 pg/g brain tissue in the ipsilateral hemisphere of sham operated mice. Mature IL-1 β levels in the contralateral hemisphere (18.4 ± 2.8 pg/g tissue) did not significantly differ from corresponding levels in sham-operated mice (14.9 ± 1.4 pg/g tissue) (Fig. 6). Elevated levels of mature IL-1 β were not detected 24 hours following injury. Early detection of ICE activity is consistent with that previously reported during apoptosis (Enari, M., *et al.*, *Nature* 380:723-726 (1996); Hara, H., *et al.*, *Proc. Natl. Acad. Sci. USA* 94:2007-2012 (1997); Hara, H., *et al.*, *J. Cereb. Blood Flow Metab.* 17:370-375 (1997)).

Example 11

ICE family inhibition reduces traumatic tissue injury.

Since ICE is activated following TBI, it was evaluated whether ICE inhibition might reduce trauma-mediated brain injury. Previously it has been demonstrated that the M17Z transgene behaves as a dominant negative inhibitor of ICE (Hara, H., *et al.*, *J. Cereb. Blood Flow Metab.* 17:370-375 (1997); Friedlander, R.M., *et al.*, *J. Exp. Med.* 185:933-940 (1997)). To evaluate whether ICE inhibition might attenuate brain trauma mediated damage, lesion size in NSE-M17Z transgenic mice with that of wild-type littermates 24 hours post-impact was compared. Total lesion volume in the NSE-M17Z mice, 24 hours following trauma, was significantly reduced by 42.3% when compared to wild-type mice (Fig. 7A). The M17Z mutant ICE gene confers tissue protection following traumatic injury, implicating ICE-like caspases as mediators of traumatic-induced cell death.

Protection from cerebral ischemia mediated injury in the NSE-M17Z transgenic mouse correlates with protection by synthetic peptide ICE family protease inhibitors (Hara, H., *et al.*, *Proc. Natl. Acad. Sci. USA* 94:2007-2012 (1997); Friedlander, R.M., *et al.*, *J. Exp. Med.* 185:933-940 (1997)). It was evaluated whether zVAD-fmk (a general ICE family protease inhibitor) would

diminish traumatic tissue damage as was demonstrated in the NSE-M17Z transgenic mouse. Wild-type mice were injected with zVAD-fmk (480ng) into the lateral cerebral ventricle 1 hour prior to impact. Total lesion volume 24 hours following trauma in the treated mice was significantly reduced by 53% when compared to the vehicle-injected mice (Fig. 7B). Moreover, lesion volume was reduced by 19% if zVAD-fmk was administered one hour following trauma (Fig. 7C). Statistical significance was only reached in the anterior two out of five slices, suggesting that a therapeutic window exists for the treatment of TBI with caspase inhibitors. These results further confirm that the caspase family plays a role in traumatic brain injury-mediated apoptosis and suggest that strategies of ICE family inhibition may be useful to treat the consequences of brain trauma.

Example 12

Free radical production is a downstream mediator of the ICE cell death cascade.

Reactive oxygen species (ROS) are potent mediators of cell death. Therefore, whether ICE-like caspases play a role in the modulation of free radical production was investigated.

Little is known regarding the actual mechanisms by which ICE activation mediates cell death. ICE can activate caspase-3, and caspase-3 has been recently shown to activate a DNase which mediates apoptotic cell death (Tewari, M., *et al.*, *Cell* 81:801-809 (1995); Enari, M., *et al.*, *Nature* 391:43-50 (1998)). Clearly additional pathways must be recruited following ICE activation playing a role mediating cell death. Free radical production has been implicated as an important downstream mediator of cell death (Greenlund, L.J.S., *et al.*, *Neuron* 14:303-315 (1995); Schulz, J.B., *et al.*, *J. Neurosci.* 16:4696-4706 (1996)). Free radical production increases following traumatic brain injury (Globus, M.Y., *et al.*, *J. Neurochem.* 65:1704-1711 (1995)). For this reason it was evaluated whether ICE inhibition *in vivo* might attenuate free radical production.

Methods

Hydroxyl radical detection. Hydroxyl radical production was determined in mice that underwent weight drop trauma, as well as in sham-operated mice. Fifteen minutes prior to craniectomy, mice were intraperitoneally injected with 400 mg 4-hydroxybenzoic acid (4-HBA)/ kg body weight, and sacrificed thirty minutes after trauma/craniectomy. Brains were removed, and the hemispheres were separated minus 2 mm of the frontal and occipital lobes. Tissue was homogenized in 0.2 M perchloric acid (1:5, w:v) at 4°C, vortexed and centrifuged (12,000 rpm, 15 min, 4°C). Supernatant was analyzed using HPLC/EC. The HPLC system consisted of a dual piston pump (ESA model 480 pump; ESA Inc., Chelmsford, MA), two pulse dampers in series, a refrigerated autosampler (CMA/200, CMA/Microdialysis) and a Coulochem II (model 5200A, ESA Inc.) electrochemical detector. Data collection was performed using an ESA501 data station. Analytes were separated on a SuperODS 5 cm x 4.6 mm, 2 mm column (TosoHaas; Montgomeryville, PA) kept at 29°C. The mobile phase delivered at 1 ml/min consisted of 100 mM NaH₂PO₄, pH 2.8 with phosphoric acid, 6.5 % methanol (v/v). Analytes were detected using dual coulometric electrode analytical cell (model 5011, ESA Inc.). The potentials applied to the first and the second electrodes were +150 mV and +700 mV, respectively. 3,4-Dihydroxybenzoic acid (3,4-DHBA) was detected on the first electrode, and 4-HBA on the second. Potential of the guard cell (model 5020, ESA Inc.), placed between the pump and the injection valve was set at +200 mV. Under these conditions, the limit of detection for 3,4-DHBA was about 1 pg on the column, and the chromatogram was completed in less than 6 min.

Results

Elevation of free radical production following trauma was significantly decreased by 43% in NSE-M17Z transgenic mice compared to its wild-type littermates. No difference of baseline free radical production was detected between sham-operated wild-type and NSE-M17Z mice (Fig. 8). The results implicate ROS production as a downstream mediator of the ICE cell death cascade.

Summary and Discussion of Examples 9-12.

These results provide evidence that ICE-like caspase-family-induced apoptosis plays an important role mediating post-traumatic cerebral damage. First, it is demonstrated that ICE activation follows traumatic injury. Second, reduced injury in a transgenic mouse expressing a dominant negative ICE inhibitor following trauma is documented. Third, it is shown that injection of a non-selective ICE-like caspase family inhibitor (zVAD-fmk) 1 hour before or 1 hour after brain trauma reduced the volume of traumatic lesion in this brain injury model. The results confirm reports indicating that apoptosis, as well as caspases, contribute to cellular injury following experimental TBI (Colicos, M.A. & Dash, P.K., *Brain Res.* 739:120-131 (1996); Yakovlev, A.G. *et al.*, *J. Neurosci.* 17:7415-7424 (1997)). Further, this data extends previous findings by implicating ICE-like caspase activation in TBI, and by demonstrating tissue protection from TBI by inhibiting the ICE cascade.

Caspases are involved in the induction and execution of programmed cell death in acute and chronic neurological disorders (Holtzman, D.M. & Deshmukh, M., *Nature Medicine* 3:954-955 (1997)). ICE itself does not appear to play a significant role in developmental apoptotic cell death as demonstrated in ICE knock-out mice (Li, P., *et al.*, *Cell* 80:401-411 (1995); Kuida, K., *et al.*, *Science* 267:2000-2003 (1995)), and in the NSE-M17Z transgenic mouse (Friedlander, R.M., *et al.*, *J. Exp. Med.* 185:933-940 (1997)). However, in view of the prominent role that ICE and ICE-like caspases play in ischemia, ALS, and trauma, it is proposed that alternate apoptotic pathways might be preferentially activated during pathological and developmental apoptosis (Hara, H., *et al.*, *Proc. Natl. Acad. Sci. USA* 94:2007-2012 (1997); Hara, H., *et al.*, *J. Cereb. Blood Flow Metab.* 17:370-375 (1997); Friedlander, R.M., *et al.*, *J. Exp. Med.* 185:933-940 (1997); Friedlander, R.M., *et al.*, *Nature* 388:31 (1997)).

There is substantial *in vitro* evidence implicating formation of ROS in certain forms of neuronal apoptosis, including those induced by staurosporine and growth

factor withdrawal (Greenlund, L.J.S., *et al.*, *Neuron* 14:303-315 (1995); Prehn, J.H.M., *et al.*, *J. Neurochem.* 68:1679-1685 (1997)). Expression of p53 induces apoptosis in hippocampal pyramidal neurons, and p53 activates genes involved in the generation of oxidative stress (Jordan, J., *et al.*, *J. Neurosci.* 17:1397-1405 (1997); Polyak, K., *et al.*, *Nature* 389:300-305 (1997)). In some forms of apoptosis, generation of ROS are an early signal in the apoptotic cascade (Greenlund, L.J.S., *et al.*, *Neuron* 14:303-315 (1995)). Recently it was shown in a BDNF deprivation model that increased peroxynitrite formation causes protein nitration, DNA fragmentation, and apoptotic cell death (Estévez, A.G., *et al.*, *J. Neurosci.* 18:923-931 (1998)). In other forms of apoptosis ROS generation is a downstream event, since ICE inhibitors block their generation (Schulz, J.B., *et al.*, *J. Neurosci.* 16:4696-4706 (1996)). The results presented above are the first to demonstrate that ICE is activated following traumatic brain injury, and that *in vivo* inhibition of the ICE cell death family reduces formation of ROS. ICE-mediated free radical formation therefore appears to be a downstream effector of caspase-induced apoptosis *in vivo*. Traumatic-induced injury, as well as cell death in other disorders featuring apoptosis, may be treated with inhibitors aimed at modulating ICE family activity to reduce brain injury and preserve brain function.

All art mentioned herein is incorporated by reference into the disclosure. Having now fully described the invention by way of illustration and example for purposes of clarity and understanding, it will be apparent to those of ordinary skill in the art that certain changes and modification may be made in the disclosed embodiments and such modifications are intended to be within the scope of the present invention. As examples, the preferred embodiments constitute only one form of carrying out the claimed invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANTS: Yuan, Junying
Friedlander, Robert M.
- (ii) TITLE OF INVENTION: Interleukin Converting Enzyme (ICE)
and Central Nervous System Damage
- (iii) NUMBER OF SEQUENCES: 28
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Sterne, Kessler, Goldstein & Fox P.L.L.C.
 - (B) STREET: 1100 New York Avenue NW, Suite 600
 - (C) CITY: Washington
 - (D) STATE: DC
 - (E) COUNTRY: USA
 - (F) ZIP: 20005-3934
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: To be assigned
 - (B) FILING DATE: Herewith
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 60/050,242
 - (B) FILING DATE: 19-JUN-1997
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Jorge A. Goldstein
 - (B) REGISTRATION NUMBER: 29,021
 - (C) REFERENCE/DOCKET NUMBER: 0609.442PC01/JAG/LBB
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (202) 371-2600
 - (B) TELEFAX: (202) 371-2540

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 402 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: not relevant
- (ii) MOLECULE TYPE: protein

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met	Ala	Asp	Lys	Ile	Leu	Arg	Ala	Lys	Arg	Lys	Gln	Phe	Ile	Asn	Ser	1	5	10	15
Val	Ser	Ile	Gly	Thr	Ile	Asn	Gly	Ile	Leu	Asp	Glu	Leu	Leu	Glu	Lys	20	25	30	
Arg	Val	Leu	Asn	Gln	Glu	Glu	Met	Asp	Lys	Ile	Lys	Leu	Ala	Asn	Ile	35	40	45	
Thr	Ala	Met	Asp	Lys	Ala	Arg	Asp	Leu	Cys	Asp	His	Val	Ser	Lys	Lys	50	55	60	
Gly	Pro	Gln	Ala	Ser	Gln	Ile	Phe	Ile	Thr	Tyr	Ile	Cys	Asn	Glu	Asp	65	70	75	80
Cys	Tyr	Leu	Ala	Gly	Ile	Leu	Glu	Leu	Gln	Ser	Ala	Pro	Ser	Ala	Glu	85	90	95	
Thr	Phe	Val	Ala	Thr	Glu	Asp	Ser	Lys	Gly	Gly	His	Pro	Ser	Ser	Ser	100	105	110	
Glu	Thr	Lys	Glu	Glu	Gln	Asn	Lys	Glu	Asp	Gly	Thr	Phe	Pro	Gly	Leu	115	120	125	
Thr	Gly	Thr	Leu	Lys	Phe	Gln	Pro	Leu	Glu	Lys	Ala	Gln	Lys	Leu	Trp	130	135	140	
Lys	Glu	Asn	Pro	Ser	Glu	Ile	Tyr	Pro	Ile	Met	Asn	Thr	Thr	Thr	Arg	145	150	155	160
Thr	Arg	Leu	Ala	Leu	Ile	Ile	Cys	Asn	Thr	Glu	Phe	Gln	His	Leu	Ser	165	170	175	
Pro	Arg	Val	Gly	Ala	Gln	Val	Asp	Leu	Arg	Glu	Met	Lys	Leu	Leu	Leu	180	185	190	
Glu	Asp	Leu	Gly	Tyr	Thr	Val	Lys	Val	Lys	Glu	Asn	Leu	Thr	Ala	Leu	195	200	205	
Glu	Met	Val	Lys	Glu	Val	Lys	Glu	Phe	Ala	Ala	Cys	Pro	Glu	His	Lys	210	215	220	
Thr	Ser	Asp	Ser	Thr	Phe	Leu	Val	Phe	Met	Ser	His	Gly	Ile	Gln	Glu	225	230	235	240
Gly	Ile	Cys	Gly	Thr	Thr	Tyr	Ser	Asn	Glu	Val	Ser	Asp	Ile	Leu	Lys	245	250	255	
Val	Asp	Thr	Ile	Phe	Gln	Met	Met	Asn	Thr	Leu	Lys	Cys	Pro	Ser	Leu	260	265	270	

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Lys Asp Lys Pro Lys Val Ile Ile Ile Gln Ala Gly Arg Gly Glu Lys
 275 280 285
 Gln Gly Val Val Leu Leu Lys Asp Ser Val Arg Asp Ser Glu Glu Asp
 290 295 300
 Phe Leu Thr Asp Ala Ile Phe Glu Asp Asp Gly Ile Lys Lys Ala His
 305 310 315 320
 Ile Glu Lys Asp Phe Ile Ala Phe Cys Ser Ser Thr Pro Asp Asn Val
 325 330 335
 Ser Trp Arg His Pro Val Arg Gly Ser Leu Phe Ile Glu Ser Leu Ile
 340 345 350
 Lys His Met Lys Glu Tyr Ala Trp Ser Cys Asp Leu Glu Asp Ile Phe
 355 360 365
 Arg Lys Val Arg Phe Ser Phe Glu Gln Pro Glu Phe Arg Leu Gln Met
 370 375 380
 Pro Thr Ala Asp Arg Val Thr Leu Ile Lys Arg Phe Tyr Leu Phe Pro
 385 390 395 400
 Gly His

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 404 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Ala Asp Lys Val Leu Lys Glu Lys Arg Lys Leu Phe Ile Arg Ser
 1 5 10 15
 Met Gly Glu Gly Thr Ile Asn Gly Leu Leu Asp Glu Leu Leu Gln Thr
 20 25 30
 Arg Val Leu Asn Lys Glu Glu Met Glu Lys Val Lys Arg Glu Asn Ala
 35 40 45
 Thr Val Met Asp Lys Thr Arg Ala Leu Ile Asp Ser Val Ile Pro Lys
 50 55 60
 Gly Ala Gln Ala Cys Gln Ile Cys Ile Thr Tyr Ile Cys Glu Glu Asp

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65	70	75	80
Ser Tyr Leu Ala Gly Thr Leu Gly Leu Ser Ala Asp Gln Thr Ser Gly	85	90	95
Asn Tyr Leu Asn Met Gln Asp Ser Gln Gly Val Ile Ser Ser Phe Pro	100	105	110
Ala Pro Gln Ala Val Gln Asp Asn Pro Ala Met Pro Thr Ser Ser Gly	115	120	125
Ser Glu Gly Asn Val Lys Leu Gln Ser Leu Glu Glu Ala Gln Arg Ile	130	135	140
Trp Lys Gln Lys Ser Ala Glu Ile Tyr Pro Ile Met Asp Lys Ser Ser	145	150	155
Arg Thr Arg Leu Ala Leu Ile Ile Cys Asn Glu Glu Phe Asp Ser Ile	165	170	175
Pro Arg Arg Thr Gly Ala Glu Val Asp Ile Thr Gly Met Thr Met Leu	180	185	190
Leu Gln Asn Leu Gly Tyr Ser Val Asp Val Lys Lys Asn Leu Thr Ala	195	200	205
Ser Asp Met Thr Thr Glu Leu Glu Ala Phe Ala His Arg Pro Glu His	210	215	220
Lys Thr Ser Asp Ser Thr Phe Leu Val Phe Met Ser His Gly Ile Arg	225	230	235
Glu Gly Ile Cys Gly Lys Lys His Ser Glu Gln Val Pro Asp Ile Leu	245	250	255
Gln Leu Asn Ala Ile Phe Asn Met Leu Asn Thr Lys Asn Cys Pro Ser	260	265	270
Leu Lys Asp Lys Pro Lys Val Ile Ile Ile Gln Ala Gly Arg Gly Asp	275	280	285
Ser Pro Gly Val Val Trp Phe Lys Asp Ser Val Gly Val Ser Gly Asn	290	295	300
Leu Ser Leu Pro Thr Thr Glu Glu Phe Glu Asp Asp Ala Ile Lys Lys	305	310	315
Ala His Ile Glu Lys Asp Phe Ile Ala Phe Cys Ser Ser Thr Pro Asp	325	330	335
Asn Val Ser Trp Arg His Pro Thr Met Gly Ser Val Phe Ile Gly Arg	340	345	350
Leu Ile Glu His Met Gln Glu Tyr Ala Cys Ser Cys Asp Val Glu Glu	355	360	365

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Ile Phe Arg Lys Val Arg Phe Ser Phe Glu Gln Pro Asp Gly Arg Ala
 370 375 380

Gln Met Pro Thr Thr Glu Arg Val Thr Leu Thr Arg Cys Phe Tyr Leu
 385 390 395 400

Phe Pro Gly His

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1320 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATGGCTGACA AGATCCTGAG GGCAAAGAGG AAGCAATTTA TCAACTCAGT GAGTATAGGG	60
ACAATAAATG GATTGTTGGA TGAACTTTGA GAGAAGAGAG TGCTGAATCA GGAAGAAATG	120
GATAAAATAA AACTTGCAAA CATTACTGCT ATGGACAAGG CACGGAACCT ATGTGATCAT	180
GTCTCTAAAA AAGGGCCCCA GGCAAGCCAA ATCTTTATCA CTTACATTG TAATGAAGAC	240
TGCTACCTGG CAGGAATTCT GGAGCTTCAA TCAGCTCCAT CAGCTGAAAC ATTTGTTGCT	300
ACAGAAGATT CTAAAGGAGG ACATCCTTCA TCCTCAGAAA CAAAGGAAGA ACAGAACAAA	360
GAAGATGGCA CATTTCAGG ACTGACTGGG ACCCTCAAGT TTTGCCCTTT AGAAAAAGCC	420
CAGAAGTTAT GGAAAGAAAA TCCTTCAGAG ATTTATCCAA TAATGAATAC AACCACCTCGT	480
ACACGTCTTG CCCTCATTAT CTGCAACACA GAGTTTCAAC ATCTTTCTCC GAGGGTTGGA	540
GCTCAAGTTG ACCTCAGAGA AATGAAGTTG CTGCTGGAGG ATCTGGGGTA TACCGTGAAA	600
GTGAAAGAAA ATCTCACAGC TCTGGAGATG GTGAAAGAGG TGAAAGAATT TGCTGCCTGC	660
CCAGAGCACA AGACTTCTGA CAGTACTTTC CTTGTATTCA TGTCTCATGG TATCCAGGAG	720
GGAATATGTG GGACCACATA CTCTAATGAA GTTTCAGATA TTTTAAAGGT TGACACAATC	780
TTTCAGATGA TGAACACTTT GAAGTGCCCA AGCTTGAAAG ACAAGCCCAA GGTGATCATT	840
ATTCAGGCAG GCCGTGGAGA GAAACAAGGA GTGGTGTGTG TAAAGATTG AGTAAGAGAC	900
TCTGAAGAGG ATTTCTTAAC GGATGCAATT TTTGAAGATG ATGGCATTAA GAAGGCCCAT	960

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ATAGAGAAAG ATTTTATTGC TTTCTGCTCT TCAACACCAG ATAATGTGTC TTGGAGACAT 1020
 CCTGTCAGGG GCTCACTTTT CATTGAGTCA CTCATCAAAC ACATGAAAGA ATATGCCTGG 1080
 TCTTGTGACT TGGAGGACAT TTTCAGAAAG GTTCGATTTT CATTGAACA ACCAGAATTT 1140
 AGGCTACAGA TGCCCACTGC TGATAGGGTG ACCCTGACAA AACGTTTCTA CCTCTTCCCG 1200
 GGACATTAAA CGAAGAATCC AGTTCATTCT TATGTACCTA TGCTGAGAAT CGTGCCAATA 1260
 AGAAGCCAAT ACTTCCTTAG ATGATGCAAT AAATATTAAA ATAAACAAA ACAGAAGGCT 1320

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 402 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Ala Asp Lys Ile Leu Arg Ala Lys Arg Lys Gln Phe Ile Asn Ser
 1 5 10 15
 Val Ser Ile Gly Thr Ile Asn Gly Leu Leu Asp Glu Leu Leu Glu Lys
 20 25 30
 Arg Val Leu Asn Gln Glu Glu Met Asp Lys Ile Lys Leu Ala Asn Ile
 35 40 45
 Thr Ala Met Asp Lys Ala Arg Asn Leu Cys Asp His Val Ser Lys Lys
 50 55 60
 Gly Pro Gln Ala Ser Gln Ile Phe Ile Thr Tyr Ile Cys Asn Glu Asp
 65 70 75 80
 Cys Tyr Leu Ala Gly Ile Leu Glu Leu Gln Ser Ala Pro Ser Ala Glu
 85 90 95
 Thr Phe Val Ala Thr Glu Asp Ser Lys Gly Gly His Pro Ser Ser Ser
 100 105 110
 Glu Thr Lys Glu Glu Gln Asn Lys Glu Asp Gly Thr Phe Pro Gly Leu
 115 120 125
 Thr Gly Thr Leu Lys Phe Cys Pro Leu Glu Lys Ala Gln Lys Leu Trp
 130 135 140

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Lys Glu Asn Pro Ser Glu Ile Tyr Pro Ile Met Asn Thr Thr Thr Arg
 145 150 155 160
 Thr Arg Leu Ala Leu Ile Ile Cys Asn Thr Glu Phe Gln His Leu Ser
 165 170 175
 Pro Arg Val Gly Ala Gln Val Asp Leu Arg Glu Met Lys Leu Leu Leu
 180 185 190
 Glu Asp Leu Gly Tyr Thr Val Lys Val Lys Glu Asn Leu Thr Ala Leu
 195 200 205
 Glu Met Val Lys Glu Val Lys Glu Phe Ala Ala Cys Pro Glu His Lys
 210 215 220
 Thr Ser Asp Ser Thr Phe Leu Val Phe Met Ser His Gly Ile Gln Glu
 225 230 235 240
 Gly Ile Cys Gly Thr Thr Tyr Ser Asn Glu Val Ser Asp Ile Leu Lys
 245 250 255
 Val Asp Thr Ile Phe Gln Met Met Asn Thr Leu Lys Cys Pro Ser Leu
 260 265 270
 Lys Asp Lys Pro Lys Val Ile Ile Ile Gln Ala Cys Arg Gly Glu Lys
 275 280 285
 Gln Gly Val Val Leu Leu Lys Asp Ser Val Arg Asp Ser Glu Glu Asp
 290 295 300
 Phe Leu Thr Asp Ala Ile Phe Glu Asp Asp Gly Ile Lys Lys Ala His
 305 310 315 320
 Ile Glu Lys Asp Phe Ile Ala Phe Cys Ser Ser Thr Pro Asp Asn Val
 325 330 335
 Ser Trp Arg His Pro Val Arg Gly Ser Leu Phe Ile Glu Ser Leu Ile
 340 345 350
 Lys His Met Lys Glu Tyr Ala Trp Ser Cys Asp Leu Glu Asp Ile Phe
 355 360 365
 Arg Lys Val Arg Phe Ser Phe Glu Gln Pro Glu Phe Arg Leu Gln Met
 370 375 380
 Pro Thr Ala Asp Arg Val Thr Leu Thr Lys Arg Phe Tyr Leu Phe Pro
 385 390 395 400
 Gly His

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1320 base pairs

-56-

(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ATGGCTGACA AGATCCTGAG GGCAAAGAGG AAGCAATTTA TCAACTCAGT GAGTATAGGG	60
ACAATAAATG GATTGTTGGA TGAACTTTTA GAGAAGAGAG TGCTGAATCA GGAAGAAATG	120
GATAAAATAA AACTTGCAAA CATTACTGCT ATGGACAAGG CACGGAACCT ATGTGATCAT	180
GTCTCTAAAA AAGGGCCCCA GGCAAGCCAA ATCTTTATCA CTTACATTTG TAATGAAGAC	240
TGCTACCTGG CAGGAATTCT GGAGCTTCAA TCAGCTCCAT CAGCTGAAAC ATTTGTTGCT	300
ACAGAAGATT CTAAAGGAGG ACATCCTTCA TCCTCAGAAA CAAAGGAAGA ACAGAACAAA	360
GAAGATGGCA CATTTCCAGG ACTGACTGGG ACCCTCAAGT TTTGCCCTTT AGAAAAAGCC	420
CAGAAGTTAT GGAAAGAAAA TCCTTCAGAG ATTTATCCAA TAATGAATAC AACCCTCGT	480
ACACGTCTTG CCCTCATTAT CTGCAACACA GAGTTTCAAC ATCTTTCTCC GAGGGTTGGA	540
GCTCAAGTTG ACCTCAGAGA AATGAAGTTG CTGCTGGAGG ATCTGGGGTA TACCGTGAAA	600
GTGAAAGAAA ATCTCACAGC TCTGGAGATG GTGAAAGAGG TGAAAGAATT TGCTGCCTGC	660
CCAGAGCACA AGACTTCTGA CAGTACTTTC CTTGTATTCA TGTCTCATGG TATCCAGGAG	720
GGAATATGTG GGACCACATA CTCTAATGAA GTTTCAGATA TTTTAAAGGT TGACACAATC	780
TTTCAGATGA TGAACACTTT GAAGTGCCCA AGCTTGAAAG ACAAGCCCAA GGTGATCATT	840
ATTCAGGCAT GCCGTGGAGA GAAACAAGGA GTGGTGTGTG TAAAAGATTG AGTAAGAGAC	900
TCTGAAGAGG ATTCTTTAAC GGATGCAATT TTTGAAGATG ATGGCATTAA GAAGGCCCAT	960
ATAGAGAAAG ATTTTATTGC TTTCTGCTCT TCAACACCAG ATAATGTGTC TTGGAGACAT	1020
CCTGTCAGGG GCTCACTTTT CATTGAGTCA CTCATCAAAC ACATGAAAGA ATATGCCTGG	1080
TCTTGTGACT TGGAGGACAT TTTCAGAAAG GTTCGATTTT CATTTGAACA ACCAGAATTT	1140
AGGCTACAGA TGCCCACTGC TGATAGGGTG ACCCTGACAA AACGTTTCTA CCTCTTCCCG	1200
GGACATTAAA CGAAGAATCC AGTTCATTCT TATGTACCTA TGCTGAGAAT CGTGCCAATA	1260
AGAAGCCAAT ACTTCCTTAG ATGATGCAAT AAATATTAAA ATAAACAAA ACAGAAGGCT	1320

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(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 402 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```

Met Ala Asp Lys Ile Leu Arg Ala Lys Arg Lys Gln Phe Ile Asn Ser
1           5           10           15

Val Ser Ile Gly Thr Ile Asn Gly Leu Leu Asp Glu Leu Leu Glu Lys
          20           25           30

Arg Val Leu Asn Gln Glu Glu Met Asp Lys Ile Lys Leu Ala Asn Ile
          35           40           45

Thr Ala Met Asp Lys Ala Arg Asn Leu Cys Asp His Val Ser Lys Lys
          50           55           60

Gly Pro Gln Ala Ser Gln Ile Phe Ile Thr Tyr Ile Cys Asn Glu Asp
          65           70           75           80

Cys Tyr Leu Ala Gly Ile Leu Glu Leu Gln Ser Ala Pro Ser Ala Glu
          85           90           95

Thr Phe Val Ala Thr Glu Asp Ser Lys Gly Gly His Pro Ser Ser Ser
          100          105          110

Glu Thr Lys Glu Glu Gln Asn Lys Glu Asp Gly Thr Phe Pro Gly Leu
          115          120          125

Thr Gly Thr Leu Lys Phe Cys Pro Leu Glu Lys Ala Gln Lys Leu Trp
          130          135          140

Lys Glu Asn Pro Ser Glu Ile Tyr Pro Ile Met Asn Thr Thr Thr Arg
          145          150          155          160

Thr Arg Leu Ala Leu Ile Ile Cys Asn Thr Glu Phe Gln His Leu Ser
          165          170          175

Pro Arg Val Gly Ala Gln Val Asp Leu Arg Glu Met Lys Leu Leu Leu
          180          185          190

Glu Asp Leu Gly Tyr Thr Val Lys Val Lys Glu Asn Leu Thr Ala Leu
          195          200          205

Glu Met Val Lys Glu Val Lys Glu Phe Ala Ala Cys Pro Glu His Lys

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210	215	220
Thr Ser Asp Ser Thr Phe Leu Val Phe Met Ser His Gly Ile Gln Glu		
225	230	235 240
Gly Ile Cys Gly Thr Thr Tyr Ser Asn Glu Val Ser Asp Ile Leu Lys		
	245	250 255
Val Asp Thr Ile Phe Gln Met Met Asn Thr Leu Lys Cys Pro Ser Leu		
	260	265 270
Lys Asp Lys Pro Lys Val Ile Ile Ile Gln Ala Gly Arg Gly Glu Lys		
	275	280 285
Gln Gly Val Val Leu Leu Lys Asp Ser Val Arg Asp Ser Glu Glu Asp		
	290	295 300
Phe Leu Thr Asp Ala Ile Phe Glu Asp Asp Gly Ile Lys Lys Ala His		
305	310	315 320
Ile Glu Lys Asp Phe Ile Ala Phe Cys Ser Ser Thr Pro Asp Asn Val		
	325	330 335
Ser Trp Arg His Pro Val Arg Gly Ser Leu Phe Ile Glu Ser Leu Ile		
	340	345 350
Lys His Met Lys Glu Tyr Ala Trp Ser Cys Asp Leu Glu Asp Ile Phe		
	355	360 365
Arg Lys Val Arg Phe Ser Phe Glu Gln Pro Glu Phe Arg Leu Gln Met		
	370	375 380
Pro Thr Ala Asp Arg Val Thr Leu Thr Lys Arg Phe Tyr Leu Phe Pro		
385	390	395 400
Gly His		

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 503 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Met Met Arg Gln Asp Arg Arg Ser Leu Leu Glu Arg Asn Ile Met Met
1 5 10 15

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Phe Ser Ser His Leu Lys Val Asp Glu Ile Leu Glu Val Leu Ile Ala
 20 25 30
 Lys Gln Val Leu Asn Ser Asp Asn Gly Asp Met Ile Asn Ser Cys Gly
 35 40 45
 Thr Val Arg Glu Lys Pro Arg Glu Ile Val Lys Ala Val Gln Arg Pro
 50 55 60
 Gly Asp Val Ala Phe Asp Ala Phe Tyr Asp Ala Leu Arg Ser Thr Gly
 65 70 75 80
 His Glu Gly Leu Ala Glu Val Leu Glu Pro Leu Ala Arg Ser Val Asp
 85 90 95
 Ser Asn Ala Val Glu Phe Glu Cys Pro Met Ser Pro Ala Ser His Arg
 100 105 110
 Arg Ser Arg Ala Leu Ser Pro Ala Gly Tyr Thr Ser Pro Thr Arg Val
 115 120 125
 His Arg Asp Ser Val Ser Ser Val Ser Ser Phe Thr Ser Tyr Gln Asp
 130 135 140
 Ile Tyr Ser Arg Ala Arg Ser Arg Ser Arg Ser Arg Ala Leu His Ser
 145 150 155 160
 Ser Asp Arg His Asn Tyr Ser Ser Pro Pro Val Asn Ala Phe Pro Ser
 165 170 175
 Gln Pro Ser Ser Ala Asn Ser Ser Phe Thr Gly Cys Ser Ser Leu Gly
 180 185 190
 Tyr Ser Ser Ser Arg Asn Arg Ser Phe Ser Lys Ala Ser Gly Pro Thr
 195 200 205
 Gln Tyr Ile Phe His Glu Glu Asp Met Asn Phe Val Asp Ala Pro Thr
 210 215 220
 Ile Ser Arg Val Phe Asp Glu Lys Thr Met Tyr Arg Asn Phe Ser Ser
 225 230 235 240
 Pro Arg Gly Met Cys Leu Ile Ile Asn Asn Glu His Phe Glu Gln Met
 245 250 255
 Pro Thr Arg Asn Gly Thr Lys Ala Asp Lys Asp Asn Leu Thr Asn Leu
 260 265 270
 Phe Arg Cys Met Gly Tyr Thr Val Ile Cys Lys Asp Asn Leu Thr Gly
 275 280 285
 Arg Gly Met Leu Leu Thr Ile Arg Asp Phe Ala Lys His Glu Ser His
 290 295 300
 Gly Asp Ser Ala Ile Leu Val Ile Leu Ser His Gly Glu Glu Asn Val

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305              310              315              320
Ile Ile Gly Val Asp Asp Ile Pro Ile Ser Thr His Glu Ile Tyr Asp
      325              330              335
Leu Leu Asn Ala Ala Asn Ala Pro Arg Leu Ala Asn Lys Pro Lys Ile
      340              345              350
Val Phe Val Gln Ala Cys Arg Gly Glu Arg Arg Asp Asn Gly Phe Pro
      355              360              365
Val Leu Asp Ser Val Asp Gly Val Pro Ala Phe Leu Arg Arg Gly Trp
      370              375              380
Asp Asn Arg Asp Gly Pro Leu Phe Asn Phe Leu Gly Cys Val Arg Pro
      385              390              395              400
Gln Val Gln Gln Val Trp Phe Lys Lys Pro Ser Gln Ala Asp Ile Leu
      405              410              415
Ile Arg Tyr Ala Thr Thr Ala Gln Tyr Val Ser Trp Arg Asn Ser Ala
      420              425              430
Arg Gly Ser Trp Phe Ile Gln Ala Val Cys Glu Val Phe Ser Thr His
      435              440              445
Ala Lys Asp Met Asp Val Val Glu Leu Leu Thr Glu Val Asn Lys Lys
      450              455              460
Val Ala Cys Gly Phe Gln Thr Ser Gln Gly Ser Asn Ile Leu Lys Gln
      465              470              475              480
Met Pro Glu Met Thr Ser Arg Leu Leu Lys Lys Phe Tyr Phe Trp Pro
      485              490              495
Glu Ala Arg Asn Ser Ala Val
      500

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(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 503 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```

Met Met Arg Gln Asp Arg Trp Ser Leu Leu Glu Arg Asn Ile Leu Glu
1           5           10           15

```

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Phe Ser Ser Lys Leu Gln Ala Asp Leu Ile Leu Asp Val Leu Ile Ala
 20 25 30
 Lys Gln Val Leu Asn Ser Asp Asn Gly Asp Met Ile Asn Ser Cys Arg
 35 40 45
 Thr Glu Arg Asp Asn Glu Lys Glu Ile Val Lys Ala Val Gln Arg Arg
 50 55 60
 Gly Asp Glu Ala Phe Asp Ala Phe Tyr Asp Ala Leu Arg Asp Thr Gly
 65 70 75 80
 His Asn Asp Leu Ala Asp Val Leu Met Pro Leu Ser Arg Pro Asn Pro
 85 90 95
 Val Pro Met Glu Cys Pro Met Ser Pro Ser Ser His Arg Arg Ser Arg
 100 105 110
 Ala Leu Ser Pro Pro Gly Tyr Ala Ser Pro Thr Arg Val His Arg Asp
 115 120 125
 Ser Ile Ser Ser Val Ser Ser Phe Thr Ser Thr Tyr Gln Asp Val Tyr
 130 135 140
 Ser Arg Ala Arg Ser Ser Ser Arg Ser Ser Arg Pro Leu Gln Ser Ser
 145 150 155 160
 Asp Arg His Asn Tyr Met Ser Ala Ala Thr Ser Phe Pro Ser Gln Pro
 165 170 175
 Ser Ser Ala Asn Ser Ser Phe Thr Gly Cys Ala Ser Leu Gly Tyr Ser
 180 185 190
 Ser Ser Arg Asn Arg Ser Phe Ser Lys Thr Ser Ala Gln Ser Gln Tyr
 195 200 205
 Ile Phe His Glu Glu Asp Met Asn Tyr Val Asp Ala Pro Thr Ile His
 210 215 220
 Arg Val Phe Asp Glu Lys Thr Met Tyr Arg Asn Phe Ser Ser Pro Arg
 225 230 235 240
 Gly Leu Cys Leu Ile Ile Asn Asn Glu His Phe Glu Gln Met Pro Thr
 245 250 255
 Arg Asn Gly Thr Lys Ala Asp Lys Asp Asn Leu Thr Asn Ile Phe Arg
 260 265 270
 Cys Met Gly Tyr Thr Val Ile Cys Lys Asp Asn Leu Thr Gly Arg Glu
 275 280 285
 Met Leu Ser Thr Ile Arg Ser Phe Gly Arg Asn Asp Met His Gly Asp
 290 295 300
 Ser Ala Ile Leu Val Ile Leu Ser His Gly Glu Glu Asn Val Ile Ile

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```

305          310          315          320
Gly Val Asp Asp Val Ser Val Asn Val His Glu Ile Tyr Asp Leu Leu
      325          330          335
Asn Ala Ala Asn Ala Pro Arg Leu Ala Asn Lys Pro Lys Leu Val Phe
      340          345          350
Val Gln Ala Cys Arg Gly Glu Arg Arg Asp Asn Gly Phe Pro Val Leu
      355          360          365
Asp Ser Val Asp Gly Val Pro Ser Leu Ile Phe Arg Gly Trp Asp Asn
      370          375          380
Arg Asp Gly Pro Leu Phe Asn Phe Leu Gly Cys Val Arg Pro Gln Val
      385          390          395          400
Gln Gln Val Trp Phe Lys Lys Pro Ser Gln Ala Asp Met Leu Ile Ala
      405          410          415
Tyr Ala Thr Thr Ala Gln Tyr Val Ser Trp Arg Asn Ser Ala Arg Gly
      420          425          430
Ser Trp Phe Ile Gln Ala Val Cys Glu Val Phe Ser Leu His Ala Lys
      435          440          445
Asp Met Asp Val Val Glu Leu Leu Thr Glu Val Asn Lys Lys Val Ala
      450          455          460
Cys Gly Phe Gln Thr Ser Gln Gly Ser Asn Ile Leu Lys Gln Met Pro
      465          470          475          480
Glu Leu Thr Ser Arg Leu Leu Lys Lys Phe Tyr Phe Trp Pro Glu Asp
      485          490          495
Arg Gly Arg Asn Ser Ala Val
      500

```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 497 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```

Met Met Arg Gln Asp Arg Arg Asn Leu Leu Glu Arg Asn Ile Leu Val
1          5          10          15

```


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Phe Ser Asn Lys Leu Gln Ser Glu Gln Ile Leu Glu Val Leu Ile Ala
 20 25 30
 Lys Gln Ile Leu Asn Ala Asp Asn Gly Asp Val Ile Asn Ser Cys Arg
 35 40 45
 Thr Glu Arg Asp Lys Arg Lys Glu Gln Val Lys Ala Val Gln Arg Arg
 50 55 60
 Gly Asp Val Ala Phe Asp Arg Phe Tyr Asp Ala Leu Arg Asp Thr Gly
 65 70 75 80
 His His Glu Leu Ala Ala Val Leu Glu Pro Leu Ala Arg Thr Asp Leu
 85 90 95
 Gly Cys Pro Met Ser Pro Ala Ser His Arg Arg Ser Arg Ala Leu Ser
 100 105 110
 Pro Ser Thr Phe Ser Ser Pro Thr Arg Val His Arg Asp Ser Val Ser
 115 120 125
 Ser Val Ser Ser Phe Thr Ser Thr Tyr Gln Asp Val Tyr Thr Arg Ala
 130 135 140
 Arg Ser Thr Ser Arg Ser Ser Arg Pro Leu His Thr Ser Asp Arg His
 145 150 155 160
 Asn Tyr Val Ser Pro Ser Asn Ser Phe Gln Ser Gln Pro Ala Ser Ala
 165 170 175
 Asn Ser Ser Phe Thr Gly Ser Ser Ser Leu Gly Tyr Ser Ser Ser Arg
 180 185 190
 Thr Arg Ser Tyr Ser Lys Ala Ser Ala His Ser Gln Tyr Ile Phe His
 195 200 205
 Glu Glu Asp Met Asn Tyr Val Asp Ala Pro Thr Ile His Arg Val Phe
 210 215 220
 Asp Glu Lys Thr Met Tyr Arg Asn Phe Ser Thr Pro Arg Gly Leu Cys
 225 230 235 240
 Leu Ile Ile Asn Asn Glu His Phe Glu Gln Met Pro Thr Arg Asn Gly
 245 250 255
 Thr Lys Pro Asp Lys Asp Asn Ile Ser Asn Leu Phe Arg Cys Met Gly
 260 265 270
 Tyr Ile Val His Cys Lys Asp Asn Leu Thr Gly Arg Met Met Leu Thr
 275 280 285
 Ile Arg Asp Phe Ala Lys Asn Glu Thr His Gly Asp Ser Ala Ile Leu
 290 295 300
 Val Ile Leu Ser His Gly Glu Glu Asn Val Ile Ile Gly Val Asp Asp

-64-

```

305          310          315          320
Val Ser Val Asn Val His Glu Ile Tyr Asp Leu Leu Asn Ala Ala Asn
          325          330          335
Ala Pro Arg Leu Ala Asn Lys Pro Lys Leu Val Phe Val Gln Ala Cys
          340          345          350
Arg Gly Glu Arg Arg Asp Val Gly Phe Pro Val Leu Asp Ser Val Asp
          355          360          365
Gly Val Pro Ala Leu Ile Phe Arg Gly Trp Asp Lys Gly Asp Gly Pro
          370          375          380
Asn Phe Leu Gly Cys Val Arg Pro Gln Ala Gln Gln Val Trp Phe Lys
          385          390          395          400
Lys Pro Ser Gln Ala Asp Ile Leu Ile Ala Tyr Ala Thr Thr Ala Gln
          405          410          415
Tyr Val Ser Trp Arg Asn Ser Ala Arg Gly Ser Trp Phe Ile Gln Ala
          420          425          430
Val Cys Glu Val Phe Ser Leu His Ala Lys Asp Met Asp Val Val Glu
          435          440          445
Leu Leu Thr Glu Val Asn Lys Lys Val Ala Cys Gly Phe Gln Thr Ser
          450          455          460
Gln Gly Ala Asn Ile Leu Lys Gln Met Pro Glu Leu Thr Ser Arg Leu
          465          470          475          480
Leu Lys Lys Phe Tyr Phe Trp Pro Glu Asp Arg Asn Arg Ser Ser Ala
          485          490          495
Val

```

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 404 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```

Met Ala Asp Lys Val Leu Lys Glu Lys Arg Lys Leu Phe Ile Arg Ser
1           5           10           15

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Met Gly Glu Gly Thr Ile Asn Gly Leu Leu Asp Glu Leu Leu Gln Thr
 20 25 30
 Arg Val Leu Asn Lys Glu Glu Met Glu Lys Val Lys Arg Glu Asn Ala
 35 40 45
 Thr Val Met Asp Lys Thr Arg Ala Leu Ile Asp Ser Val Ile Pro Lys
 50 55 60
 Gly Ala Gln Ala Cys Gln Ile Cys Ile Thr Tyr Ile Cys Glu Glu Asp
 65 70 75 80
 Ser Tyr Leu Ala Gly Thr Leu Gly Leu Ser Ala Asp Gln Thr Ser Gly
 85 90 95
 Asn Tyr Leu Asn Met Gln Asp Ser Gln Gly Val Ile Ser Ser Phe Pro
 100 105 110
 Ala Pro Gln Ala Val Gln Asp Asn Pro Ala Met Pro Thr Ser Ser Gly
 115 120 125
 Ser Glu Gly Asn Val Lys Leu Gln Ser Leu Glu Glu Ala Gln Arg Ile
 130 135 140
 Trp Lys Gln Lys Ser Ala Glu Ile Tyr Pro Ile Met Asp Lys Ser Ser
 145 150 155 160
 Arg Thr Arg Leu Ala Leu Ile Ile Cys Asn Glu Glu Phe Asp Ser Ile
 165 170 175
 Pro Arg Arg Thr Gly Ala Glu Val Asp Ile Thr Gly Met Thr Met Leu
 180 185 190
 Leu Gln Asn Leu Gly Tyr Ser Val Asp Val Lys Lys Asn Leu Thr Ala
 195 200 205
 Ser Asp Met Thr Thr Glu Leu Glu Ala Phe Ala His Arg Pro Glu His
 210 215 220
 Lys Thr Ser Asp Ser Thr Phe Leu Val Phe Met Ser His Gly Ile Arg
 225 230 235 240
 Glu Gly Ile Cys Gly Lys Lys His Ser Glu Gln Val Pro Asp Ile Leu
 245 250 255
 Gln Leu Asn Ala Ile Phe Asn Met Leu Asn Thr Lys Asn Cys Pro Ser
 260 265 270
 Leu Lys Asp Lys Pro Lys Val Ile Ile Ile Gln Ala Cys Arg Gly Asp
 275 280 285
 Ser Pro Gly Val Val Trp Phe Lys Asp Ser Val Gly Val Ser Gly Asn
 290 295 300
 Leu Ser Leu Pro Thr Thr Glu Glu Phe Glu Asp Asp Ala Ile Lys Lys

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```

305              310              315              320
Ala His Ile Glu Lys Asp Phe Ile Ala Phe Cys Ser Ser Thr Pro Asp
      325              330              335
Asn Val Ser Trp Arg His Pro Thr Met Gly Ser Val Phe Ile Gly Arg
      340              345              350
Leu Ile Glu His Met Gln Glu Tyr Ala Cys Ser Cys Asp Val Glu Glu
      355              360              365
Ile Phe Arg Lys Val Arg Phe Ser Phe Glu Gln Pro Asp Gly Arg Ala
      370              375              380
Gln Met Pro Thr Thr Glu Arg Val Thr Leu Thr Arg Cys Phe Tyr Leu
      385              390              395              400
Phe Pro Gly His

```

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 171 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```

Met Leu Thr Val Gln Val Tyr Arg Thr Ser Gln Lys Cys Ser Ser Ser
1              5              10              15
Lys His Val Val Glu Val Leu Leu Asp Pro Leu Gly Thr Ser Phe Cys
      20              25              30
Ser Leu Leu Pro Pro Pro Leu Leu Leu Tyr Glu Thr Asp Arg Gly Val
      35              40              45
Asp Gln Gln Asp Gly Lys Asn His Thr Gln Ser Pro Gly Cys Glu Glu
      50              55              60
Ser Asp Ala Gly Lys Glu Glu Leu Met Lys Met Arg Ile Pro Thr Arg
      65              70              75              80
Ser Asp Met Ile Cys Gly Tyr Ala Cys Leu Lys Gly Asn Ala Ala Met
      85              90              95
Arg Asn Thr Lys Arg Gly Ser Trp Tyr Ile Glu Ala Leu Thr Gln Val
      100              105              110

```

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Phe Ser Glu Arg Ala Cys Asp Met His Val Ala Asp Met Leu Val Lys
 115 120 125
 Val Asn Ala Leu Ile Lys Glu Arg Glu Gly Tyr Ala Pro Gly Thr Glu
 130 135 140
 Phe His Arg Cys Lys Glu Met Ser Glu Tyr Cys Ser Thr Leu Cys Gln
 145 150 155 160
 Gln Leu Tyr Leu Phe Pro Gly Tyr Pro Pro Thr
 165 170

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 5 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Gln Ala Cys Arg Gly
 1 5

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 33 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

AAGTCGACGC CATGGCTGAC AAGATCCTGA GGG

33

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 33 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

AAGTCGACGC CATGAACAAA GAAGATGGCA CAT

33

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AAGTCGACGC CATGGGCATT AAGAAGGCC ATA

33

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TTCCCGGGTC ATCTTCAAAA ATTGCATCCG

30

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 36 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

AACCCGGGAG GCCTCCATGA TCGTCAAGA TAGAAG

36

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

AACCCGGGAC GGCAGAGTTT CGTGCTTCCG

30

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

ATTCAGGCCT CCAGAGGAGA GAAAC

25

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GGCAGGATTC TCAGCATAGG T

21

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(2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 28 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CAAGGCCTGC CTGAATAATG ATCACCTT

28

(2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 36 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GCAGGCCTGT CGATCGGAAC GTCGTGACAA TGGATT

36

(2) INFORMATION FOR SEQ ID NO:23:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

ACAGGCCTGC ACAAAAACGA TTTT

24

(2) INFORMATION FOR SEQ ID NO:24:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs

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- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

TGCCCCAAGCT TGAAAGACAA GCCC

24

(2) INFORMATION FOR SEQ ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 22 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CTGGCGAAAG GGGGATGTGC TG

22

(2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 4 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 1
- (D) OTHER INFORMATION: /product= "OTHER"

/label= Ac

/note= "An acetyl (Ac) group is attached to the tyrosine residue."

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 4
- (D) OTHER INFORMATION: /product= "OTHER"

/label= CMK

/note= "A chloromethylketone (CMK) group is attached to the

-72-

aspartic acid residue."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Tyr Val Ala Asp
1

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 4 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 1
- (D) OTHER INFORMATION: /product= "OTHER"

/label= z

/note= "An N-benzyloxycarbonyl (z) group is attached to the N-terminal aspartic acid residue."

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 4
- (D) OTHER INFORMATION: /product= "OTHER"

/label= FMK

/note= "A fluoromethylketone (FMK) group is attached to the C-terminal aspartic acid residue."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Asp Glu Val Asp
1

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 4 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: peptide

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 1

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(D) OTHER INFORMATION: /product= "OTHER"
/label= Ac
/note= "An acetyl (Ac) group is attached to the N-terminal
tyrosine residue."

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 4
(D) OTHER INFORMATION: /product= "OTHER"
/label= CHO
/note= "An aldehyde (CHO) is attached to the C-terminal
aspartic acid residue."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

Tyr Val Ala Asp
1

What Is Claimed Is:

1. A method of treating ALS or ALS-like symptoms comprising inhibiting ICE by gene therapy.

5 2. The method of claim 1, wherein said gene therapy uses a mutant *ICE* gene, said gene encoding the amino acid sequence of SEQ. ID. No. 1 or SEQ. ID. No. 2 in Figure 1A-1B.

3. The method of claim 1, wherein said gene therapy uses a mutant gene encoding an amino acid change in the active site of ICE.

10 4. The method of claim 3, wherein said mutant gene is a mouse gene that encodes a C284G mutant ICE.

5. The method of claim 3, wherein said mutant gene is found in pJ655 (ATCC accession no. 209077).

15 6. The method of claim 3, wherein said mutant gene is a human gene that encodes a C285G mutant ICE.

7. The method of claim 2, wherein said gene is a degenerate variant of the mutant *ICE* gene encoding the amino acid sequence of SEQ ID. Nos. 1 or SEQ. ID. No. 2 in Figure 1.

20 8. The method of claim 1, wherein said gene therapy uses a mutant *ICE* gene, said gene having the DNA sequence of Figure 2D (SEQ ID. No. 3).

9. The method of claim 8, wherein said gene comprises a degenerate variant of the DNA sequence of Figure 2D (SEQ ID. No. 3).

10. A method for modulating programmed cell death accompanying ALS comprising contacting a cell with modulating amounts of the mutant *ICE* gene product.
- 5 11. A transgenic non-human animal comprising a mutant *ICE* gene and a mutant *SOD* gene.
12. The transgenic non-human animal as claimed in claim 9, wherein said animal is a mouse.
13. The transgenic non-human animal as claimed in claim 8, wherein said transgenic animal has attenuated ALS symptoms.
- 10 14. A transgenic non-human animal model for the study of ALS wherein said model comprises an animal with a mutant *ICE* gene and a mutant *SOD* gene.
- 15 15. The progeny of said transgenic non-human animal claimed in claim 9.
16. A method of screening compounds for treating ALS, comprising:
- 15 (a) providing a transgenic non-human animal having a mutant *ICE* gene and a mutant *SOD* gene, said transgenic animal exhibiting attenuated symptoms of ALS;
- (b) administering a compound to be tested to said transgenic animal;
- 20 (c) determining the effect of said compound on ALS; and
- (d) correlating the effect of said compound on ALS in said animal with the effect of said compound on animals with said mutant *SOD* gene but without said mutant *ICE* gene.

17. A method of obtaining a new transgenic non-human animal having a mutant *ICE* gene and a mutant *SOD* gene, said transgenic animal characterized by attenuated ALS symptoms, said method comprising:

- 5 a) mating a first transgenic animal having a mutant *ICE* gene to a second transgenic animal having a mutant *SOD* gene;
- b) obtaining DNA from the progeny of said mating; and
- c) verifying that the genotype of said progeny contains the mutant *ICE* and *SOD* genes.

10 18. The progeny of the transgenic animals obtained by the method of claim 13.

15 19. A method for delaying mortality from ALS comprising treating subjects having ALS or ALS symptoms with inhibitors of ICE selected from the group consisting of N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD.FMK), acetyl-Tyr-Val-Ala-Asp-chloromethylketone (Ac-YVAD.CMK), N-benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone (z-DEVD.FMK) and Ac-YVAD-CHO.

20 20. A method for attenuating or preventing apoptosis resulting from traumatic brain injury comprising treating a patient in need of such treatment by inhibiting an ICE-like caspase.

 21. A method for reducing the formation of reactive oxygen species following brain trauma comprising treating a patient in need of such by inhibiting an ICE-like caspase.

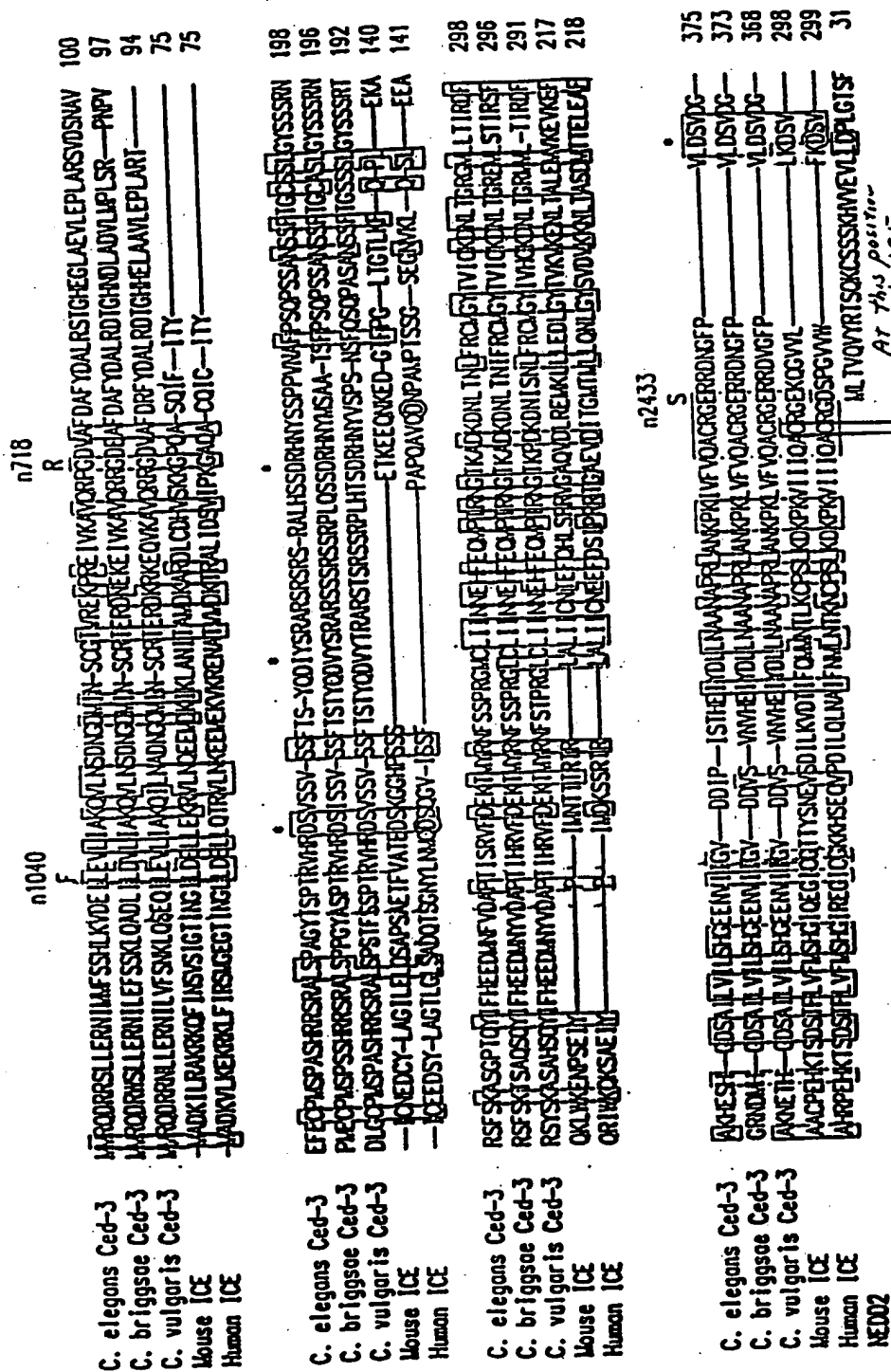


FIG. 1A

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n1129, n1164

C. elegans Ced-3
C. briggsae Ced-3
C. vulgaris Ced-3
Mouse ICE
Human ICE
NEED2

VPALF---RQMDNRG---PLFNF LGCVRPQV---QQWFFKK---FSQADILIRVAITIAQVSHRVSARGSHF---ICAVEVFSITAAQDOWELLTEVKK 464
VPSLI---FRGMDNRG---PLFNF LGCVRPQV---QQWFFKK---FSQADILIRVAITIAQVSHRVSARGSHF---ICAVEVFSITAAQDOWELLTEVKK 462
VPALI---FRGMDNRG---P---NF LGCVRPQA---QQWFFKK---FSQADILIRVAITIAQVSHRVSARGSHF---ICAVEVFSITAAQDOWELLTEVKK 455
-----ROSEE---DELTDAIFED---DGI-KKA-HIEKQ---TAFCSIPDNVSHRPVPGSD---IESL IKHKEVANSQDLEDFRKYRF- 373
-----GVSGNL---SLPTTEEFED---QAI-KKA-HIEKQ---TAFCSIPDNVSHRPVPGSD---IGRL IETAEVACSDVEEIFRKYRF- 375
CSLLPPPLLLEYTORQV---GQCKKNTQSFGCESDACKELMKARIT---TRESMIGVYALDKGNAARNITKRGSWITIEAL TQVSEFACDIPVADAILVKVNAL 131

n2430 n2426 n1163

V
C. elegans Ced-3
C. briggsae Ced-3
C. vulgaris Ced-3
Mouse ICE
Human ICE
NEED2

VA---QCFPTSGCSNII---QCFPTSGCSNII---KFFYTFEAR---NSAV 503
VA---QCFPTSGCSNII---QCFPTSGCSNII---KFFYTFEDRG RNSAV 503
VA---QCFPTSGCSNII---QCFPTSGCSNII---KFFYTFEDRN RSSAV 496
---SFE-QCFEFLQIPTADVT---KFFYTFESH--- 402
---SFE-QCFEFLQIPTADVT---KFFYTFESH--- 404
IKEREGAPCTEFHRCGSGSILCOQMLFEG---YPPT 171

FIG. 1B

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FIG. 2A

MADKILRAKRKQFINSV SIGTINGLLDELLEKRVLNQEEM 40
DKIKLANITAMDKARNLCDHVSKKGPQASQIFITYICNED 80
CYLAGILELQSAPSAETVATEDSKGGHPSSSETKEEQNK 120
EDGTFPGLTGTCLKFCPLEKAQKLWKENPSEIYPIMNTTTR 160
TRLALICNTEFQHLSPRVGAQVDLREMKLLEDLGYTVK 200
VKENLTALEMVKEVKEFAACPEHKTS DSTFLVFM SHGIQE 240
GICGTTYSNEVSDILKVD TIFQMMNTLKCP SLKDKPKVII 280
IQACRGEKQGVVLLKDSVRDSEEDFLTDAIFEDDGIKKAH 320
IEKDFIAFCSSTPDNVSWRHPVRGSLFIESLIKHMKEYAW 360
SCDLEDIFRKVRFSFEQPEFRLQMPTADRVTLTKRFYLP 400
GH.

FIG. 2B

atggctgacaagatcctgagggcaagaggaagcaattta 40
tcaactcagtgagtatagggacaataatggattgttga 80
tgaacttttagagaagagagtgctgaatcaggaagaaatg 120
gataaaataaaaacttgcaaacattactgctatggacaagg 160
cacggaacctatgtgatcatgtctctaaaaagggcccca 200
ggcaagccaaatctttatcacttacatttgaatgaagac 240
tgctacctggcaggaaattctggagcttcaatcagctccat 280
cagctgaaacatttgttgctacagaagattctaaaggagg 320
acatccttcacctcagaacaaaggaagacagaacaaa 360
gaagatggcacatttcaggactgactgggacctcaagt 400
tttgccttttagaaaaagcccagaagttatggaaagaaa 440
tccttcagagatttatccaataatgaataaccactcgt 480
acacgtcttgccctcattatctgcaacacagatttcaac 520
atctttctccgaggggttgagctcaagtgacctcagaga 560
aatgaagttgctgctggaggaictgggtataccgtgaaa 600
gtgaaagaaaatctcacagctctggagatggtgaaagagg 640
tgaaagaatttgctgctgcccagagcacaagacttctga 680
cagtactttccttgattcatgtctcatggtatccaggag 720
ggaatatgtgggaccacatactctaataagtttcagata 760
tttaaaaggttgacacaatctttcagatgatgaacacttt 800
gaagtgcccaagcttgaagacaagcccaagtgatcatt 840
attcaggcatgccgtggagagaacaaggagtggtgtgt 880
taaaagattcagtaagagactctgaagaggatttctaac 920
ggatgcaatttttgaagatgatggcattaagaaggcccat 960
atagagaaagattttatgtcttctgctcttcaacaccag 1000
ataatgtgtcttgagacatcctgtcaggggctcacttt 1040
cattgagtcactcatcaaacacatgaagaatatgcctgg 1080
tctgtgacttgaggacatttcagaaaggttcgatttt 1120

FIG. 2B (CONT'D)

cattgaacaaccagaatttaggctacagatgccactgc 1160
tgatagggtgaccctgacaaaacgtttctacctcttcccg 1200
ggacattaaacgaagaatccagttcattcttatgtaccta 1240
tgctgagaatcgtgccaaataagaagccaatacttccttag 1280
atgatgcaataaatattataaaataaaacaaaacagaaggct 1320

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FIG. 2C

MADKILRAKRKQFINSV SIGTINGLLDELLEKRVLNQEEM 40
DKIKLANITAMDKARNLCDHVSKKGPQASQIFTTYICNED 80
CYLAGILELQSAPSAET FVATEDSKGGHPSSSETKEEQNK 120
EDGTFPGLTGTLKFCPLEKAQKLWKENPSEIYPIMNTTTR 160
TRLALICNTEFQHLSPRVGAQVDLREMKLLLEDLG YTVK 200
VKENLTAL EMVKEVKEFAACPEHKTSDSTFLVFM SHGIQE 240
GICGTTYSNEVSDILKVD TIFQMMNTLKCPSLKDKPKVII 280
IQAGRGEKQGVVLLKDSVRDSEEDFLTDAIFEDDGIKKAH 320
IEKDFIAFCSSTPDNVSWRHPVRGSLFIESLIKHMKEYAW 360
SCDLEDIFRKVRFSFEQPEFRLQMPTADRVTLTKRFYLP 400
GH.

FIG. 2D

atggctgacaagatcctgagggcaagaggaagcaattatcaactcagtgagtataggg 60
acaataaatggattgttgatgaacttttagagaagagagtgctgaatcaggaagaaatg 120
gataaaataaaacttgcaaacttactgctatggacaaggcacggaacctatgtgatcat 180
gtctctaaaaaaggccccaggcaagccaaatctttatcacttacatttgaatgaagac 240
tgctacctggcaggaattctggagcttcaatcagctccatcagctgaacatttgttgc 300
acagaagattctaaaggaggacatccctcactcagaaacaaaggagaacagaacaaa 360
gaagatggcacatttcaggactgactgggaccctcaagtttgcctttagaaaagcc 420
cagaagttatggaaagaaaatccttcagagatttatccaataatgaatacaaccactcgt 480
acacgtcttgcctcattatctgcaacacagagtttcaacatctttccaggagggttga 540
gtcgaagttgacctcagagaatgaagttgctgctggaggatctgggtataccgtgaaa 600
gtgaaagaaaatctcacagctctggagatggtgaaagaggtgaaagaatttgcctgc 660
ccagagcacaagacttctgacagtactttcctgtattcatgtctcatggtatccaggag 720
ggaatatgtgggaccacatacttcaatgaagttcagatattttaagggtgacacaatc 780
tttcagatgatgaacatttgaagtgcccaagcttgaaagacaagcccaaggtgatcatt 840
attcaggcaggccgtggagagaaacaaggagtggtgtgttaaaagattcagtaagagac 900
tctgaagaggatttcttaacgggatgcaattttgaagatgatggcattaagaagcccat 960
atagagaaagattttattgcttctgctctcaacaccagataatgtgtcttgagacat 1020
cctgtcaggggctcattttcattgagtcactcatcaaacacatgaagaatatgcctgg 1080
tctgtgacttgaggacattttcagaaagggtcgattttcatttgaacaaccagaatt 1140
aggctacagatgccactgctgatagggtgaccctgacaaaacgtttctaccttcccg 1200
ggacattaaacgaagaatccagttcattcttatgtacctatgctgagaatcgtgccaata 1260
agaagccaatacttccctagatgatgcaataaatattaaaaataaaaacaaacagaaggct 1320

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3A

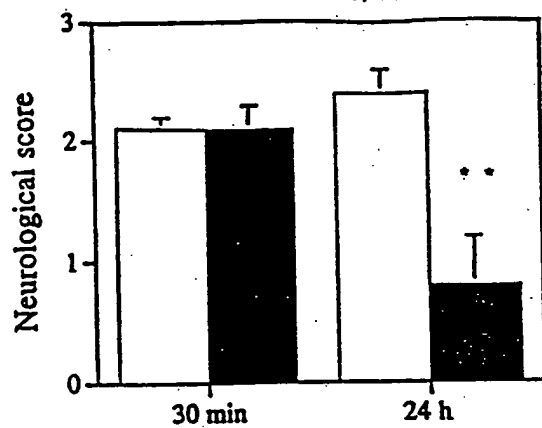
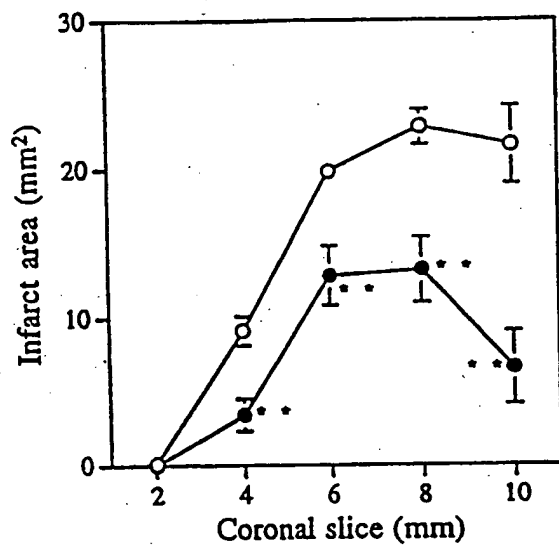
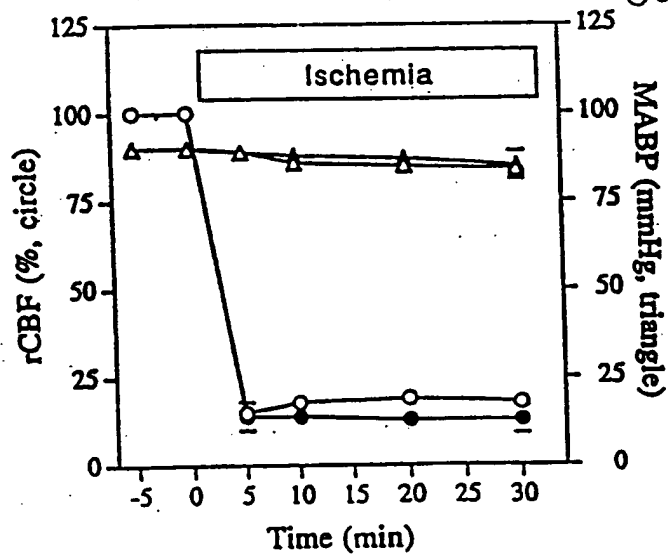


FIG 3A-3C

3B



3C



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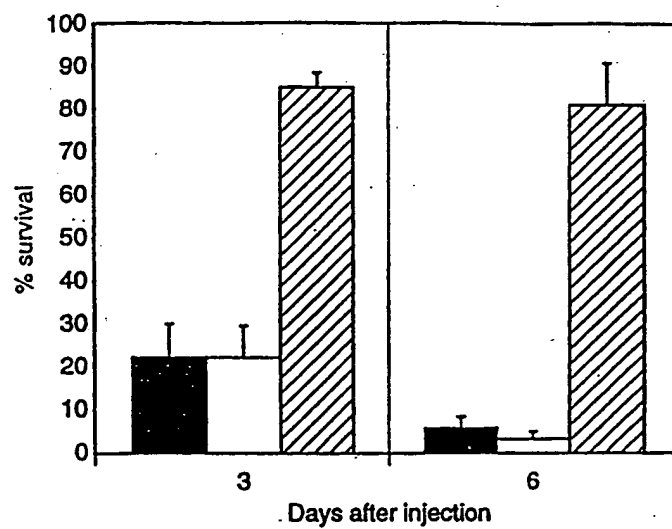
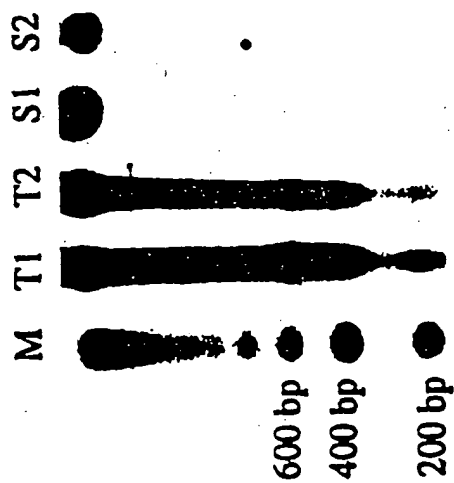


FIG 4.

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FIGURE 5



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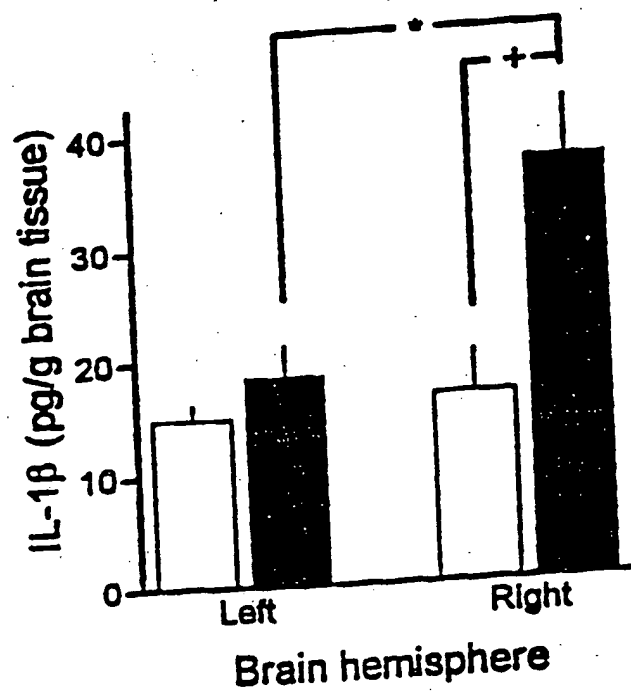
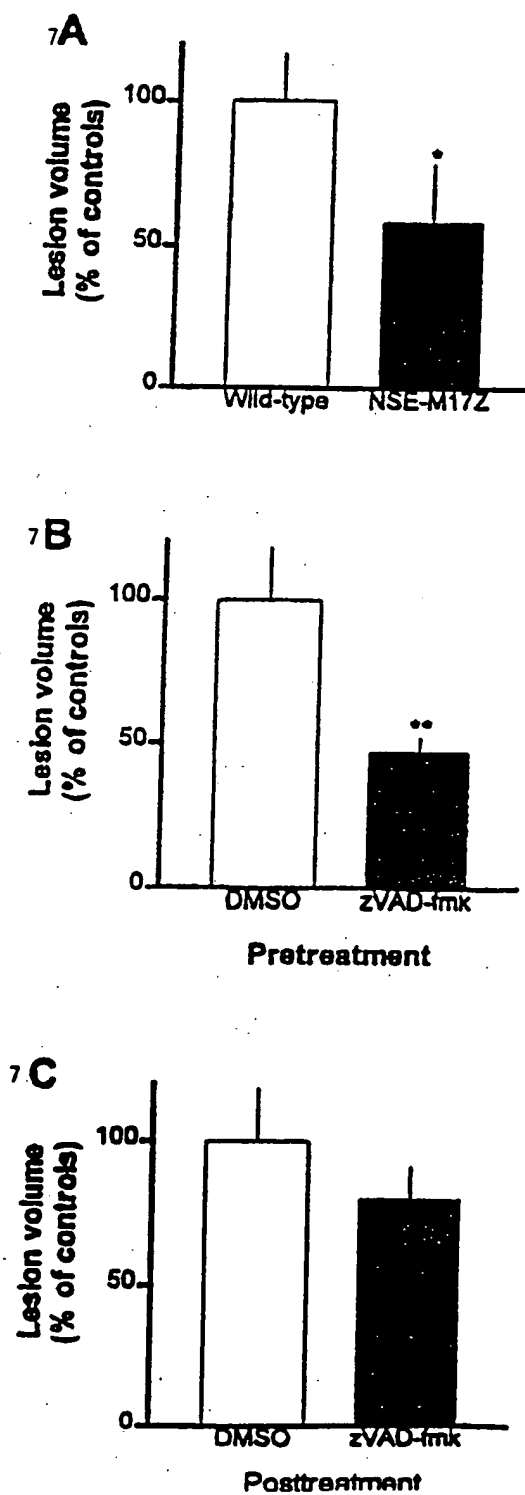


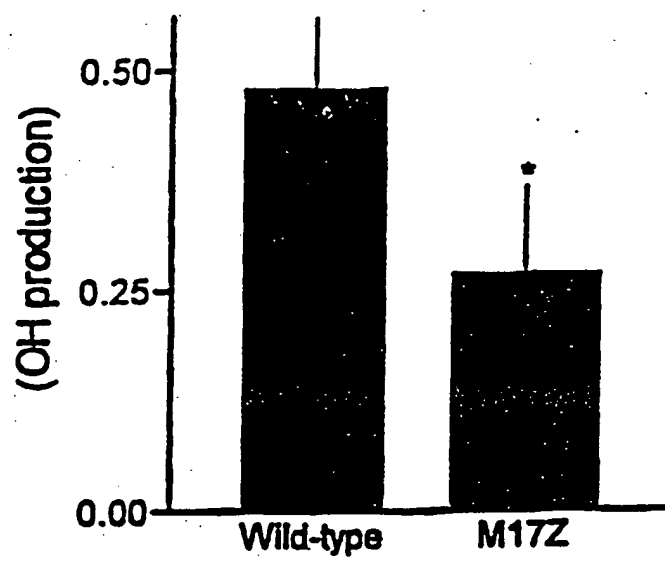
FIGURE 6

FIGURE 7A-7C



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FIGURE 8



3,4 DHBA / 4 HSA
p-methyl / m-methyl

Applicant's or agent's file reference number 0609.442PC01	International application No. PCT/US 98/12716
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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM
(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>11</u> , line <u>18</u> .	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution AMERICAN TYPE CULTURE COLLECTION	
Address of depositary institution (including postal code and country) 10801 University Blvd. Manassas, Virginia 20110-2209 United States of America	
Date of deposit 28 May 1997 (28.05.97)	Accession Number 209077
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
DNA Plasmid pJ655 In respect of those designations in which a European Patent is sought a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample (Rule 28(4) EPC).	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (If the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the international Bureau later (specify the general nature of the indications, e.g. "Accession Number of Deposit")	

<p align="center">For receiving Office use only</p> <p><input checked="" type="checkbox"/> This sheet was received with the international application</p> <p>Authorized officer <i>Elena Rivera</i> Patrol Specialist MPO - PCT Operations (703) 305-2578</p>	<p align="center">For international Bureau use only</p> <p><input type="checkbox"/> This sheet was received by the international Bureau on:</p> <p>Authorized officer</p>
---	---

CANADA

The applicant hereby requests that, until either a Canadian patent has been issued on the basis of the application or the application has been refused, or is abandoned and no longer subject to reinstatement, or is withdrawn, the furnishing of a sample of deposited biological material referred to in the application only be effected to an independent expert nominated by the Commissioner of Patents.

DENMARK

The applicant hereby requests that, until the application has been laid open to public inspection (by the Danish Patent Office), or has been finally decided upon by the Danish Patent Office without having been laid open to public inspection, the furnishing of a sample shall only be effected to an expert in the art. The request to this effect shall be filed by the applicant with the Danish Patent Office not later than at the time when the application is made available to the public under Sections 22 and 33(3) of the Danish Patents Act. If such a request has been filed by the applicant, any request made by a third party for the furnishing of a sample shall indicate the expert to be used. That expert may be any person entered on a list of recognized experts drawn up by the Danish Patent office or any person approved by the applicant in the individual case.

FINLAND

The applicant hereby requests that, until the application has been laid open to public inspection (by the National Board of Patents and Registration), or has been finally decided upon by the National Board of Patents and Registration without having been laid open to public inspection, the furnishing of a sample shall only be effected to an expert in the art. The request to this effect shall be filed by the applicant with the International Bureau before the expiration of 16 months from the priority date (preferably on the Form PCT/RO/134 reproduced in annex Z of Volume I of the PCT Applicant's Guide). If such a request has been filed by the applicant, any request made by a third party for the furnishing of a sample shall indicate the expert to be used. That expert may be any person entered on a list of recognized experts drawn up by the National Board of Patents and Registration or any person approved by the applicant in the individual case.

NETHERLANDS

The applicant hereby requests that until the date of a grant of a Netherlands patent or until the date on which the application is refused or withdrawn or lapsed, the microorganism shall be made available as provided in Rule 31F(1) of the Patent Rules only by the issue of a sample to an expert. The request to this effect must be furnished by the applicant with the Netherlands Industrial Property Office before the date on which the application is made available to the public under Section 22C or Section 25 of the Patents Act of the Kingdom of the Netherlands, whichever of the two dates occurs earlier.

SWEDEN

The applicant hereby requests that, until the application has been laid open to public inspection (by the Swedish Patent Office), or has been finally decided upon by the Swedish Patent Office without having been laid open to public inspection, the furnishing of a sample shall only be effected to an expert in the art. The request to this effect shall be filed by the applicant with the International Bureau before the expiration of 16 months from the priority date (preferably on the Form PCT/RO/134 reproduced in annex Z of Volume I of the PCT Applicant's Guide). If such a request has been filed by the applicant, any request made by a third party for the furnishing of a sample shall indicate the expert to be used. That expert may be any person entered on a list of recognized experts drawn up by the Swedish Patent office or any person approved by the applicant in the individual case.

UNITED KINGDOM

The applicant hereby requests that the furnishing of a sample of a microorganism shall only be made available to an expert. The request to this effect must be filed by the applicant with the International Bureau before the completion of the technical preparations for international publication of the application.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/12716**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : A61K 48/00; A61K 38/43; C12N 15/00

US CL : 424/94.1; 435/172.3; 514/44; 800/2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/94.1; 435/172.3; 514/44; 800/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FRIEDLANDER et al. Expression of a dominant negative mutant of interleukin-1-beta converting enzyme in transgenic mice prevents neuronal cell death induced by trophic factor withdrawal and ischemic brain injury. J. Exp. Med. 03 March 1997, Vol. 185, No. 5, pages 933-940, entire document.	1-11, 14, 16-17, 19-21
Y, P	FRIEDLANDER et al. Inhibition of ICE slows ALS in mice. Nature. 03 July 1997, Vol. 388, page 31, entire document.	1-11, 14, 16-17, 19-21
Y - X	HARA et al. Inhibition of interleukin-1-beta converting enzyme family proteases reduces ischemic and excitotoxic neuronal damage. Proc. Natl. Acad. Sci. USA. March 1997, Vol. 94, pages 2007-2012, entire document.	1-11, 14, 16-17, 20-21 19

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but aimed to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
B earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

03 AUGUST 1998

Date of mailing of the international search report

03 SEP 1998

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

ANNE-MARIE BAKER, PH.D.

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/12716

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GURNEY et al. Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. Science. 17 June 1994, Vol. 264, pages 1772-1775, entire document.	1-11, 14, 16-17, 19-21
Y	NETT et al. Molecular cloning of the murine IL-1-beta converting enzyme cDNA. J. Immunol. 15 November 1992, Vol. 149, No. 10, pages 3254-3259, entire document.	1-11, 14, 16-17, 19-21
X,P ----- Y,P	YAKOVLEV et al. Activation of CPP32-like caspases contributes to neuronal apoptosis and neurological dysfunction after traumatic brain injury. J. Neurosci. 01 October 1997, Vol. 17, No. 19, pages 7415-7424.	20 ----- 1-11, 14, 16-17, 19-21
Y	CERRETTI et al. Molecular characterization of the gene for human interleukin-1-beta converting enzyme (IL1BC). Genomics. 1994, Vol. 20, pages 468-473, entire document.	1-11, 14, 16-17, 19-21

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/12716

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: 12, 13, 15, and 18
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Please See Extra Sheet.
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/12716

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, STN file medicine

search terms: ice, interleukin converting enzyme, amyotrophic, head trauma, (thi or traumatic brain injury), programmed cell death, apoptosis, caspase, caspase-1, CASP-1, pro-interleukin-1beta, ICE and gene therapy, ICE and transgen?, mutant(3a)ice, ICE inhibitors, protease inhibitors, dominant negative, (SOD or superoxide dismutase), (ROS or reactive oxygen species), treatment(5a)ALS, (amyotrophic or ALS)

BOX 1. OBSERVATIONS WHERE CLAIMS WERE FOUND UNSEARCHABLE

2. Where no meaningful search could be carried out, specifically:

Claims 12 and 15 refer to the transgenic non-human animal claimed in claim 9, but claim 9 is drawn to a method of ICE-inhibiting gene therapy and not a transgenic animal. Claim 13 refers to the transgenic non-human animal claimed in claim 8, but claim 8 is again drawn to a method of ICE-inhibiting gene therapy. Claim 18 is drawn to the progeny of the transgenic animals obtained by the method of Claim 13, however, Claim 13 is drawn to a transgenic animal and not a method for obtaining a transgenic animal.